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INHIBITION OF GROWTH OF STRAINS OF *SALMONELLA TYPHIMURIUM* BY *MESO*-TARTRATE

G A Alfredsson and D C Old

Division of Biology, University of Iceland, Reykjavik, Iceland, and Bacteriology Department, University of Dundee, Dundee, Scotland

When strains of *Salmonella typhimurium* unable to utilize *meso* tartrate were grown for 24 hours in peptone water containing 0 1 or 1 per cent of *meso* tartrate, measurements of the optical densities of the cultures were considerably less than those of cultures grown in peptone water without *meso* tartrate This reduction in the growth yield was observed not only when the *meso* tartrate was present throughout the whole period of incubation, but also when the *meso* tartrate was added to cultures in the log phase of growth, for there was then an immediate reduction in the rate of growth and a reduction in the final population of cells These effects were observed whether growth was assessed turbidimetrically or by viable count The effect of the *meso* tartrate was not bactericidal, for washing of the inhibited cells and their transfer to fresh peptone water released them from the inhibited state Some Krebs cycle intermediates, such as oxaloacetate, malate, fumarate or succinate, when added to peptone water cultures of *meso* tartrate non fermenting strains stimulated their growth and when added together with the *meso* tartrate prevented the *meso* tartrate from exerting its growth inhibitory effect Other Krebs intermediates, such as citrate, although growth stimulatory, had only a limited effect in releasing cells from the growth inhibition by *meso* tartrate Inhibition was also observed in defined salts media in which the presence of *meso* tartrate prevented *S typhimurium* strains from *meso* tartrate negative biotypes from using glycerol or citrate as sole carbon source

acid and the three isomers of tartaric re of major importance in the Kristen-irhoff scheme for the biotyping of of *Salmonella typhimurium* (8, 9) The ty to ferment one or more of these is characteristic of strains from nine 21 biotypes recognised in that scheme cteria unable to ferment, for example, tartrate* are not adversely affected ultured in media in the presence of ite (2) In the course of a biotyping on the general use of organic acids in the biotyping of strains of *S typhimurium* (2, 3) we noticed that the presence of *meso*-tartrate not only failed to stimulate the growth of bacteria from *meso*-tartrate-non-fermenting biotypes but also inhibited their growth This paper reports further observations on the phenomenon

MATERIALS AND METHODS

Bacteria

Wild type strains of *Salmonella typhimurium* were from the series examined by *Duguid et al* (7) and other strains from diverse sources received from Dr *E S Anderson*, Enteric Reference Laboratory, Colindale, London The biotypes of the

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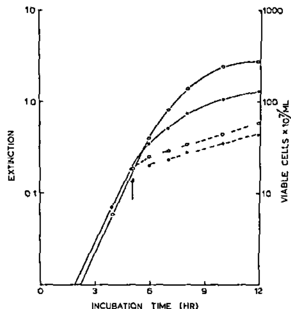


Fig 1 The influence of *meso* tartrate on the growth of the *meso* tartrate negative strain S2633 of *Salmonella typhimurium* At 5 hours (arrow), the shaken peptone water culture in log phase was divided into two aliquots, one of which received *meso* tartrate (final concentration, 0 1 per cent) and the other distilled water Incubation was continued at 37°C and samples removed for optical density and viable count measurements Optical density (Spekker), control ●——●, with *meso* tartrate ● --● Viable count ($\times 10^7$ bacteria/), control O——O, with *meso* tartrate O --O

ture of the *meso* tartrate negative strain of *S typhimurium* S2633 After 5 hr incubation, when the optical density of the culture was 0 18 (Spekker units) and the viable cell count was $2\ 09 \times 10^8$ bacteria/ml, the culture was divided into two aliquots to one of which *meso*-tartrate was added Both the optical density measurements and the viable counts made after the addition of the *meso* tartrate showed that there was an immediate reduction in the growth rate of the culture grown in the presence of *meso* tartrate Although there was in the *meso* tartrate containing culture a continued exponential increase in both viable cells and in optical density at this concentration of *meso* tartrate, 1 mg/ml the rate of increase was considerably less than that in the control peptone water culture Fig 2 reveals that the extent of the inhibition of growth was dependent on the concentration of *meso*-tartrate in the culture In many other experiments of this kind, we noticed that the strong inhibitory effects observed on the addition of 1 mg/ml *meso* tartrate to log-phase cultures gradually disappeared so that at 24 hr there was little difference in either optical density or viable counts Thus, it seemed that there could be a slow, gradual release from the inhibited state in the later stages of growth

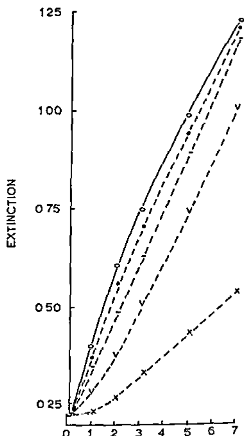


Fig 2 The influence of different concentrations of *meso* tartrate on the growth (optical density) of the *meso* tartrate negative strain S2294 of *Salmonella typhimurium* After five hours incubation at 37°C (0) the shaken peptone water cultures had optical densities of *c* 0 24 (Spekker) and the following additions were made distilled water (*i e* control) O——O *meso* tartrate at the following concentrations, x- -x 0 05 per cent v v 0 01 per cent / --/0 005 per cent, ●---● 0 001 per cent Incubation was continued to 12 hours, *i e* 7 hours after the additions had been made

Although this was never observed in experiments of the kind reported in Table 1, it should be noted that in these experiments the *meso* tartrate concentration was ten times higher at 10 mg/ml and present from the beginning of cultivation

Release from Inhibition by Removal of Meso *Tartrate*

Fig 3 shows that removal of the *meso*-tartrate from a peptone water culture of strain S2317 by washing of the inhibited cells (A4) in fresh peptone water released the inhibition of growth with a corresponding shift in the growth rate to that of the control cultures (A1, A3) that had never been in contact with *meso* tartrate

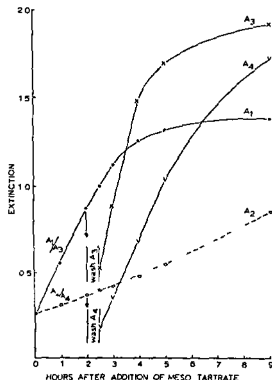


Fig 3 The release of the inhibition of growth by *meso* tartrate by washing The *meso* tartrate negative strain S2317 of *Salmonella typhimurium* cultured in peptone water and aerated by shaking for 5 hours (0) was divided into four aliquots two of which received 0 1 per cent *meso* tartrate (A2 and A4) and two of which did not (A1 and A3) After a further two hours incubation to 7 hours (2), one of the control non inhibited cultures (A3) and one of the inhibited cultures (A4) were washed and resuspended in fresh peptone water Incubation was continued to 14 hours (9) and samples were removed for optical density measurements (Spekker)

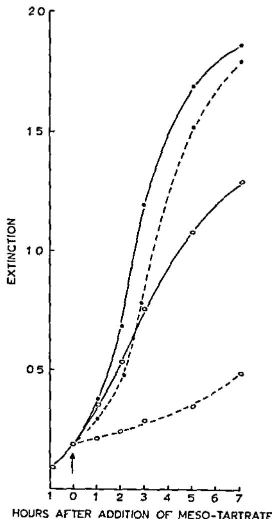


Fig 4 The ability of succinate to release *meso* tartrate inhibition of growth. Four flasks containing peptone water were inoculated identically with the *meso* tartrate negative strain S2633 of *Salmonella typhimurium* and samples removed until 5 hours when the optical density (Spekker) of all cultures was 0 18 ± 0 005 To the flasks the following additions were made distilled water (*i e* control) O——O *meso* tartrate (final concentration of 0 1 per cent) O-- O succinate (0 1 per cent) and distilled water ●——●, succinate (0 1 per cent) and *meso* tartrate (0 1 per cent) ●---● Incubation was continued at 37°C with aeration of the cultures. The curves are drawn from the optical density readings over the first 12 hours only

Prevention of Inhibition of Growth by Addition of Metabolites

We investigated the effects of the addition of different metabolites to log-phase peptone water cultures at the same time as the *meso*-tartrate Figs 4 and 5 show the outcome of a series of experiments in which some Krebs cycle intermediates were examined All the substrates were tested for their ability to prevent the growth-inhibitory effects of *meso*-tartrate at 1 mg/ml in peptone water cultures at 5 hr In Fig 4, the intermediate examined was succinate which was shown (1) to stimulate the growth of *S typhimurium* S2633 when added alone to the peptone water culture and (2) to release *meso*-tartrate inhibition after a very short lag period so that the growth rate and yield of the culture that received both the *meso*-tartrate and the succinate was as great as that to which only succinate had been added The stimulatory effect and the inhibition releasing abilities of the other three intermediates tested are shown in Fig 5, in which it can be seen (comparison of curves B, C and D with A) that oxaloacetate, malate and fumarate stimulated growth and released the cells from their inhibited state as efficiently as succinate had done Similar experiments, not reported here, suggest that citrate, although growth stimulatory for strain S2633, was much less effective than oxaloacetate, malate, fumarate or succinate in releasing the growth inhibition by *meso* tartrate α Ketoglutarate neither stimulated growth nor released the *meso*-tartrate inhibition, probably due to its impermeability in strain S2633

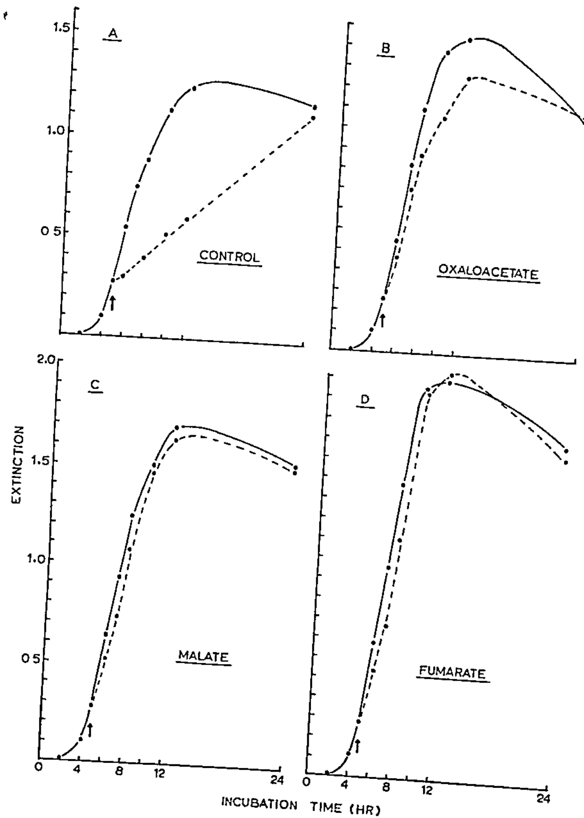
Effects in Defined Media

Inhibition of growth by *meso* tartrate was demonstrable in defined as well as in peptone media For example, most of the 1435 strains of *S typhimurium* in our series were able to utilize citrate as sole carbon source in minimal salts medium and grew well on citrate minimal agar within 48 hr (3) In the presence of *meso* tartrate, this ability to utilize citrate was severely inhibited and no growth was found in 48 hr although with longer periods of incubation mutant growth appeared All the strains whose ability to utilize citrate as sole carbon source was inhibited by the presence of *meso*-tartrate were from biotypes 4, 12 and 16 in the Kristensen Harhoff scheme and other groups of strains that belonged to *meso*-tartrate negative biotypes not recognised by previous workers (2, 3) Growth inhibition by *meso* tartrate was also severe on glycerol minimal medium and, again, the inhibited strains were those whose ability to utilize citrate had also been inhibited The use of glucose as sole carbon source however, was unaffected by the presence of *meso* tartrate and strains from both *meso* tartrate-positive and *meso* tartrate negative biotypes grew well on glucose minimal agar either in the absence or in the presence of *meso tartrate*

DISCUSSION

There are very few reports of the inhibition of bacterial enzymes or systems by *meso* tartrate apart from the inhibition of *d tartrate* and l *tartrate* dehydrases of *Pseudomonas* (16, 17) An early report of the inhibition of the lactate and succinate dehydrogenases of *Escherichia coli* by *meso* tartrate is also known (15) More recently, it has been shown that concentrations of tartaric acid from 0 01–0 1 per cent partially or completely inhibited the oxidation of nitrite by whole cells of *Nitrobacter agilis* (20) The inhibiting isomer of tartaric acid was not named but the concentration used to achieve this inhibition was similar to that reported

Fig 5 The influence of oxaloacetate fumarate and malate on the growth and the inhibition of growth by *meso* tartrate of the *meso* tartrate negative strain S2633 of *Salmonella typhimurium* Oxaloacetate (B), malate (C) and fumarate (D), final concentrations of 0 1 per cent were added at 5 hours (↑) alone ●——● or together with *meso* tartrate (0 1 per cent) ●– – –● to peptone water cultures that were aerated with shaking (A) represents control peptone water cultures to which only water ●——● or *meso* tartrate (0 1 per cent) ● – –● was added



A
B
C
D
CONTROL
OXALOACETATE
MALATE
FUMARATE
1.5
1.0
0 5
2.0
1.5
1.0
0 5
0
4
8
12
24
EXTINCTION
INCUBATION TIME (HR)

here for the inhibition of growth of *Salmonella typhimurium*. This inhibitory effect of *meso*-tartrate was also reported by *Lewis & Stocker* (11) who investigated *S typhimurium* strains of phage type 1a/2 and found that 15 per cent of them were *meso-tartrate* sensitive, i e strains that grew well on defined solid media, especially with citrate as carbon source, did not grow or grew poorly when *meso* tartrate was also present The results reported in this paper and those of *Alfredsson et al* (3) confirm and extend their findings and show that *meso*-tartrate sensitivity (or inhibition of growth by *meso*-tartrate) is common to all but a few strains from all *meso* tartrate non-fermenting biotypes and to bacteria of many phage types *Lewis & Stocker* (11) had also demonstrated that this sensitivity to *meso*-tartrate was dependent on the carbon source, being consistent and complete when the carbon source was citrate, glycerol, maltose or lactose, but neither complete nor consistent when the carbon source was glucose, galactose or glucose plus citrate Their experiments included attempts at relieving the inhibition and most interesting was their observation that the addition of as little as 0 01 per cent of casein hydrolysate to the media on which the *meso* tartrate sensitivity was severe often brought about a release from inhibition The addition of most kinds of amino acids, either singly or in combination, even up to 0 1 per cent, did not reverse the inhibition, whereas the addition of glutamate, proline or histidine to the minimal medium usually reversed the inhibition by 48 hr though seldom by 18 hr

Aspartate, among all the amino acids tested, allowed a significant, though incomplete reversal by 18 hr and complete growth by 48 hr In other experiments not reported in this paper (2), we too found aspartate to be an effective releaser of *meso* tartrate inhibition in log-phase bacteria in peptone water *Lewis & Stocker* assigned the efficiency of these amino acids as reversers to the readiness with which *S typhimurium* strains used them as sources of carbon and energy, although they also noted that the reversal efficiencies did not correlate with their efficiences as substrates for growth

Our findings add further to the list of substrates that effectively release cells from their inhibited state, for we have found that succinate, malate, oxaloacetate and fumarate were most effective and citrate partially effective It is certainly conceivable that all the substrates act as releasers of inhibition because they open up new avenues for energy production and biosynthesis in the presence of the *meso*-tartrate The ineffectiveness of citrate itself may indicate that its use as a substrate for growth under these sparing conditions involves a *meso*-tartrate sensitive step for citrate added alone to peptone water grown cultures in the absence of *meso* tartrate stimulated growth

In non bacterial systems, several enzymes associated with the Krebs cycle are known to be inhibited by *meso* tartrate For example, in bovine heart mitochondrial system, *meso* tartrate inhibits competitively the oxidation of *L* malate by malate dehydrogenase this enzyme is also capable of slowly oxidizing *meso*-tartrate (and *laevo* tartrate) to oxalo glycolate (5, 10) Both the conversion of malate into pyruvate by the malic enzyme (decarboxylating) of pigeon liver (18) and the synthesis of malate from pyruvate by the same enzyme (19) are *meso* tartrate sensitive, and *meso*-tartrate is one of the most potent competitive inhibitors known for pig heart fumarase (1, 12) With rat kidney slices, a 70 per cent inhibition of pyruvate oxidation was seen with 20 mM *meso* tartrate *but not* by the *D* or *DL* tartrates This inhibition could be reversed by fumarate or to a lesser extent by malate or citrate (14) However with rat liver or kidney mitochondria, the most striking inhibition was that of the oxidation of α ketoglutarate (14) We are currently investigating in more detail the processes of *meso*-tartrate inhibition and its reversal by addition of different metabolites to log phase cultures in defined liquid medium Our results so far suggest that the site of inhibition of *S typhimurium* may also be a step in the Krebs cycle, perhaps isocitrate dehydrogenase

One of us (*GAA*) thanks the *Icelandic Science Foundation* and *NATO* for generous financial sup port during the tenure of this work We thank Professor *J P Duguid* for his advice and valuable discussions

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STUDIES ON MANNITOL-FERMENTING STRAINS OF *STREPTOCOCCUS BOVIS*

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Altogether 94 strains of the so called mannitol fermenting type of *Str bovis* have been studied These strains have been found to form a very homogeneous group of streptococci, culturally and biochemically They differ clearly from the *Str bovis* described by *Orla Jensen* It is suggested that these streptococci should be considered as identical with the *Str faecalis septicus* of *Friedberg*

Str bovis was described by *Orla Jensen* (1919) and (1943) and found particularly characteristic of bovine faeces

According to *Orla Jensen*, *Str bovis* is primarily characterized by its rather narrow temperature interval of growth (22°–45° C), its ability to form capsules and its pronounced ability to ferment starch With good sources of nitrogen, inulin and raffinose are fermented too, whereas mannitol (or other alcohols) are never fermented Moreover, *Orla Jensen* paid attention to the ability of *Str bovis* to ferment arabinose

The strains examined by *Orla-Jensen* (1919) all grew well in milk and hydrolyzed casein This property was not concistently found with the freshly isolated strains examined later, *Orla-Jensen* (1943)

From sour milk as well as from bovine faeces, *Orla-Jensen* isolated another group of streptococci, having a certain resemblance to *Str bovis* These streptococci, for which he proposed the name *Str inulinaceus*, seemed more apt to ferment inulin than *Str bovis* but were not starch-fermenters to the same degree From *Str bovis* they differed first of all in not forming capsules and in having a less narrow temperature interval of growth Moreover, fermentation of mannitol was found characteristic of *Str inulinaceus*, whereas arabinose was not fermented, *Orla Jensen* did not examine his strains of *Str bovis* or *Str inulinaceus* as to haemolysis on blood agar

Apparently neglecting the importance attached by *Orla Jensen* to the ability of Str bovis to form capsules and inability to ferment mannitol, *Sherman* (1937) concluded that there was little justification for considering *Str inulinaceus* as anything more than a variant of *Str bovis*, and in his later paper *Sherman* (1938) stated that a substantial proportion of strains of *Str bovis* do ferment mannitol This point of view has generally been accepted till now

Slime formation on sucrose agar was originally recognized by *Niven et al* (1941) with several strains of *Str bovis* The slime

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was identified by *Bailey & Oxford* (1958) as a dextran, and the presence of CO was found by *Dain et al* (1956) to have a profound effect on slime formation

The property of slime formation was regarded by *Seeley & Dain* (1960) as one of the most important taxonomical characteristics of *Str bovis*, and *Dain et al* (1956) stated that the slime forming strains were generally mannitol non fermenting and α haemolytic in contrast to the mannitol fermenting, not slime producing strains

Strains of *Str bovis* have been isolated from animal as well as from human sources Strains of bovine origin have been found frequently to belong to the mannitol non fermenting α haemolytic type, whereas strains of human origin have more often been mannitol fermenting, γ streptococci, *Niven et al* (1948), *Dain et al* (1956), *Seeley & Dain* (1960) and *Medrek & Barnes* (1962) In this connection it may be pertinent to draw attention to the strains of non haemolytic streptococci studied by *Friedberg* (1941) for which he proposed the species name *Str faecalis septicus* These streptococci were principally deliminated on the basis of their ability to ferment mannitol, salicin aesculin and inulin *Friedberg* did not examine his strains as to fermentation of starch but representatives of *Friedberg's* original strains were later studied by *Skadhauge* (1950) and *Seelemann* (1954) and found to be starch fermenting The *Str faecalis septicus* of Friedberg showed much resemblance to *Str inulinaceus* of Orla Jensen but was regarded by *Seelemann* (1954) as representatives of the mannitol fermenting variant of *Str bovis*

Serologically mannitol fermenting as well as mannitol non fermenting strains, designated as *Str bovis*, have been found to possess the group D antigen of Lancefield, *Shattock* (1949), *Jones & Shattock* (1960) Analysis of type antigens has been carried out by *Medrek & Barnes* (1962), who set up altogether 11 different serological types of mannitol non fermenting strains based on the presence of capsular antigens

Deibel (1964) concluded that the slime forming mannitol non fermenting strains of *Str bovis* form a taxanomic entity, whereas the mannitol fermenting strains, which do not produce slime, are not clearly defined

It has been the purpose of the present investigations to analyse a fairly large number of mannitol fermenting strains of *Str bovis* as to cultural and biochemical properties in order to elucidate the relationship of these strains to *Str bovis* and *Str inulinaceus* of Orla Jensen as well as to *Str faecalis septicus* of Friedberg

MATERIALS AND METHODS

The material comprises altogether 94 strains of mannitol fermenting streptococci primarily classified as *Str bovis* Out of the 94 strains 84 have been isolated from human cases of bacteriaemia Two strains originated from human faeces and guinea pig respectively Six strains are subcultures of the original strains of *Str faecalis septicus* of Friedberg Finally the serological type strains C 101 of Medrek & Barnes and the ATCC strain 9809 were included

For comparison 9 strains of the typical mannitol non fermenting type of Str bovis were examined namely the serological type strains of Medrek & Barnes C 3 , C 36 , C 48 S2/5b and S 63 , the ATCC strain 15351 and 3 strains of bovine origin isolated by the authors (K 3 K 10 and K 34)

All strains have been examined as to the following properties Morphology, formation of capsules haemolysis on 5 per cent horse blood agar, presence of catalase motility, growth on 40 per cent bile blood agar growth in filtered beef broth containing 6 5 per cent NaCl resistance to sodium tellurite (1 2500) production of NH_3 from arginine slime formation on 5 per cent sucrose agar and fermentation of glucose maltose lactose, sucrose, raffinose inulin salicin aesculin starch mannitol, sorbitol glycerol arabinose, trehalose, melibiose and melezitose using Difco's phenol red broth base with one per cent of the drugs

In addition part of the strains were examined as to slime formation on 5 per cent sucrose agar in a 10 per cent CO_2 atm ability to grow at 10°, 20°, 22° and 45° C coagulation of milk, hydrolysis of casein on 0 1 per cent peptone agar to which had been added 5 per cent of skim milk and growth on thallium acetate tetrazolium agar (T A T)

TABLE 1 *Physiological Characteristics of* Streptococcus bovis *Strains from Different Sources*

Origin of the strains	Number of str	Growth on blood agar	Capsules	Fermentation of					
				Starch	Raffinose	Mannitol	Inulin	Arabinose	Trehalose
Human cases of septicaemia	79	γ	—	+	+	+	+	—	+
Human cases of septicaemia	5	γ	—	+	+	+	—	—	+
Human faeces	1	γ	—	+	+	+	+	—	+
Guinea pig	1	γ	—	+	+	+	+	—	+
Friedberg's Str faecalis sept	5	γ	—	+	+	+	+	—	+
Str faecalis sept	1	β	—	+	+	+	+	—	+
Medrek & Barnes Strain C 101	1	γ	—	+	+	+	+	—	+
ATCC 9809	1	γ	—	+	+	+	+	—	+
Cow dung K 3	1	α	+	+	+	—	+	+	—
Cow dung K 10	1	α	+	+	+	—	—	+	—
Cow dung K 34	1	α	+	+	+	—	—	+	—
Medrek & Barnes Strains S 63	1	α	+	+	+	—	+	+	+
S/2/5b	1	α	+	+	+	—	+	—	—
C 48	1	α	+	+	+	—	+	—	—
C 3	1	α	+	+	+	—	—	+	—
C 36	1	α	+	+	+	—	—	+	—
ATCC 15351	1	α	+	+	+	—	+	—	—

RESULTS

Properties common to all strains Gram-positive cocci occurring in pairs or short chains, growth on 40 per cent bile-blood-agar, no growth on sodium tellurite-agar, no growth in broth containing 6 5 per cent of NaCl All the strains were non-motile, did not form catalase or produce NH_3 from arginine Fermentation of glucose, maltose, lactose, sucrose raffinose, salicin, aesculin, starch and melibiose No fermentation with sorbitol, glycerol or melizitose

Properties of mannitol-fermenting and mannitol-non-fermenting strains respectively As is evident from Table 1, all but one of the *mannitol-fermenting strains were γ-streptococci* The one [s]train showing β-haemolysis was a representat[ive o]f Friedberg's *Str faecalis septicus* wh[ich ap]parently has changed from γ- to β-hae[molysi]s [d]uring storage and subculturing Non[-...] the mannitol-fermenting strains formed capsules Inulin-fermentation was lacking in only 5 out of the 94 mannitol-fermenting strains, and none of these strains fermented arabinose

The mannitol-non-fermenting strains, on the contrary, all showed α haemolysis and capsule formation As to fermentation of inulin and arabinose, these strains differed, some strains fermented, some did not

As shown in Table 2, coagulation of milk and hydrolysis of casein are characteristic properties of the mannitol-fermenting strains in contrast to the mannitol-non-fermenti[ng] ones Slime-production in the presence of a was found with 56 out of 84 mannitol fer[menting] strains, but with only 2 of the [9] mannitol-non-fermenting strains examined In the presence of 10 per cent CO , 68 out of 84 mannitol-fermenting strains were found to produce slime 4 out of the 9 mannitol-non fermenting strains were shown to produce slime in the presence of 10 per cent

TABLE 2 *Additional Characteristics of Mannitol and Mannitol Non Fermenting Strains of* Streptococcus *bovis* Compared with Streptococcus faecalis septicus

Reaction	Mannitol fermenting strains of *Kiel & Skadhauge*	Strains of *Friedberg's Str faecalis septicus*	Mannitol non fermenting strains of *Str bovis*
Coagulation of milk	55/55	5/5	0/5
Hydrolysis of casein	82/84	5/5	0/9
Slime production in air	56/84	3/6	2/9
Slime production in 10 per cent CO_2	68/84	4/6	4/9
White colonies (TAT agar)	47/48	4/5	1/5
Growth at			
10° C	0/55	0/6	0/7
20° C	26/52	1/6	0/8
22° C	42/51	6/6	0/8
45° C	57/61	6/6	7/8

TAT agar — Talliumacetat tetrazolium agar

CO Generally the mannitol fermenting strains grew on TAT agar with white colonies while 4 out of 5 mannitol non fermenting strains showed pink colonies As to temperature interval of growth most of the *mannitol fermenting strains were able to* grow at 20° or 22° C whereas this was not found to be the case with any of the mannitol non fermenting strains studied Nearly all strains grew at 45° C No strains grew at 10° C

DISCUSSION

As is evident from the results of this investigation the mannitol fermenting *strains* characterized by their further ability to ferment starch inulin and raffinose culturally and biochemically form a rather homogeneous group showing resemblances to the *Str inulinaceus* of Orla Jensen as well as to the *Str faecalis septicus* of Friedberg These strains differ from the *Str inulinaceus* in their failing ability to ferment melezitose and hydrolyse casein The difference in ability to attack casein can possibly be due to the use of different methods *Orla Jensen found* no casein hydrolysis if Str inulinaceus were grown in milk and the amount of solubilized casein were determined Unfortunately it has not been possible to the authors to have strains of Orla Jensen s original collection of *Str inulinaceus* at disposal for comparison It is doubtfull whether such strains are available at all and therefore no definite conclusions can be drawn as to the identity of the mannitol fermenting strains with *Str inulinaceus*

In contrast to the findings by *Dain et al* (1956) slime production was found in our *investigations to be characteristic of* most of the mannitol fermenting strains in the presence of air as well as in air with 10 per cent CO

Freshly isolated mannitol non fermenting strains *from cow dung all formed slime in the* presence of 10 per cent CO whereas most of the museum strains have lost this ability

The mannitol fermenting strains differ from *Str bovis of Orla Jensen* not only in fermenting mannitol but first of all in their missing ability to form capsules and as to the temperature interval of growth properties which should probably not be underestimated in a discussion on taxonomy

Our results thus are fully in agreement with the statement of *Medrek & Barnes* (1962), that at least two main divisions are present within the species *Str bovis* The

mannitol-fermenting strains of *Str bovis* should probably be regarded as identical with *Str faecalis septicus* of Friedberg

We would like to thank Mrs A L Kronman for her valuable assistance

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ELECTRON MICROSCOPY OF *TREPONEMA CUNICULI*

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Treponema cuniculi was studied in the electron microscope by means of negative staining and ultrathin sectioning techniques The sectioned organisms were studied *in situ* in biopsies from lesions of infected skin Cells of *T cuniculi* were found to be regular helices 8 15 µm long and 0 15 µm wide The ends of the cells were pointed and three flagella were inserted in a row subterminally at each end The flagella wound around the organisms and over lapped in the middle of the treponemes The fine structure of isolated flagella was similar to that of flagella obtained from other treponemes Treatment of *T cuniculi* with *Myxo bacter* AL-1 protease I revealed bundles of intracytoplasmic microtubules The diameter of each tubule was about 7 nm In the ultrathin sections of infected tissue *T cuniculi* was observed in intercellular spaces in the *stratum spinosum* and the *stratum basale* layers of the epidermis No treponemes were observed in the connective tissue of the dermis Sec tions of the microorganisms show the same structural pattern as previously described for *T pallidum* Nichols. The organisms are surrounded by two tripple layered membranes with the flagella situated in between The ultrastructure of the pathogenic treponemes *T cuni culi* *T pallidum* and *T pertenue* is compared

Treponema cuniculi is the cause of venereal spirochetosis of rabbits It was first mentioned in 1912 by *Ross* (22) The first description of these treponemes was published by *Bayon* (1) in 1913 He described them as having tapered ends without terminal flagella In 1920 *Jacobsthal* (11) suggested the name *Spirochaeta paralues cuniculi* for this organ ism In a paper published 1921 *Noguchi* (17) changed the name to *Treponema cuniculi* A review on the current status of *T cuniculi* was given by *Smith & Pesetsky* (26) in 1967

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All reports on the morphology of *T cuni culi* stress the fact that this treponeme can not be differentiated from the human patho gene *Treponema pallidum* and these two organisms also cross react serologically Pre vious studies on the morphology of *T cuni culi* have been carried out by light micro-scopy To the best of our knowledge no studies on the ultrastructure of *T cuniculi* have been published as yet, apart from a brief report from this laboratory (9) pre senting the preliminary results of the present study For this reason we considered it worth while to extend our studies on *T cuniculi* in order to obtain more data for a comparison with previous studies on *T pallidum* (6 12, 14) Our aim was to determine if a differ entiation of these two organisms could be

achieved on the basis of their ultrastructure The results of these efforts are presented in this paper

MATERIALS AND METHODS

Treponema cuniculi infections are prevalent at the moment, among adult rabbits in our rabbit colony Superficial and limited lesions occur on the skin and the mucous membranes of the genitalia The treponemes were obtained from scrotal skin lesions because preliminary tests had shown that the specimens obtained from these lesions were the least contaminated by other microorganisms

Preparations for Negative Staining

Scaly patches were rubbed intensively and carefully with a cotton swab moistened in a salt solution containing sucrose (SMC*) Especial care was taken to avoid bleeding A preparation for examination in the light microscope was made by squeezing the cotton swab into a small drop of either SMC or 3 per cent glutaraldehyde in SMC on a microscope slide This was then examined in the light microscope by dark field illumination to see if the number of treponemes was sufficient for preparation of specimens for electron microscopy A minimum number of about 3×10^7 organisms per ml was necessary but contamination with a high number of blood cells and/or a large amount of cell debris could be a limiting factor

If the preparation seemed satisfactory the cover glass was removed carefully, and a drop of 1 per cent ammonium molybdate adjusted to pH 7 with NH_4OH was added and mixed with the drop on the slide Formvar coated carbon reinforced grids were placed film side down on top of the drop To make the surface charge more suitable for an even distribution of material on the film the grids had previously been exposed to an ultraviolet light source (25) After 30–60 seconds the grids were removed from the drop and the liquid sucked off carefully with the edge of a piece of filter paper After drying the grids were ready for examination in the electron microscope

Some treponemes were disintegrated by treatment on specimen grids with detergents or with *Myxobacter* AL 1 protease 1** The detergents used were 0 2 per cent Teepol or 1 per cent sodium deoxycholate, both in redistilled water The enzyme was used in concentrations of 100 µg/ml or 200 µg/ml in 5×10^{-4} M EDTA in $2 5 \times 10^{-3}$ M TRIS buffer, pH 9 0 The procedure used was the multiple drop technique described in an earlier paper (8) The time of treatment with the active agents varied between 1 and 10 minutes

Preparations for Sectioning

Small pieces of skin with lesions were taken from the scrotum of a rabbit and immersed directly in a formaldehyde glutaraldehyde mixture (12) After fixation for about 30 minutes, the biopsies were cut into small pieces of about 1 mm^3, and fixation in fresh fixative was continued for another 1–1½ hours The tissue blocks were then washed twice in 0 2 M sucrose in 0 1 M cacodylate buffer, pH 7 2, containing 0 01 M $CaCl_2$ and stored overnight at 4° C in the same buffer The next day they were post fixed for 1 hour at room temperature in 1 per cent OsO_4 in veronal acetate buffer pH 7 3, containing 0 01 M $CaCl_2$ and 4 5 per cent sucrose (4) After a brief wash in the same buffer without sucrose the biopsies were treated for 1 hour at room temperature with 2 per cent uranyl acetate in the same buffer (28), dehydrated in alcohol and propylene oxide (16) and finally embedded in Vestopal W (23) or in Spurr's low viscosity epoxy mixture (27)

Ultrathin sections were cut on the LKB ultrotome III microtome and post stained with magnesium uranyl acetate (5) and lead citrate (21)

Electron Microscopy

Electron microscopy was carried out on a Philips EM 200 or a Philips EM 300 electron microscope at primary magnifications of *1500 9000* and 16000 × Negatives were obtained on Kodak *Fine*

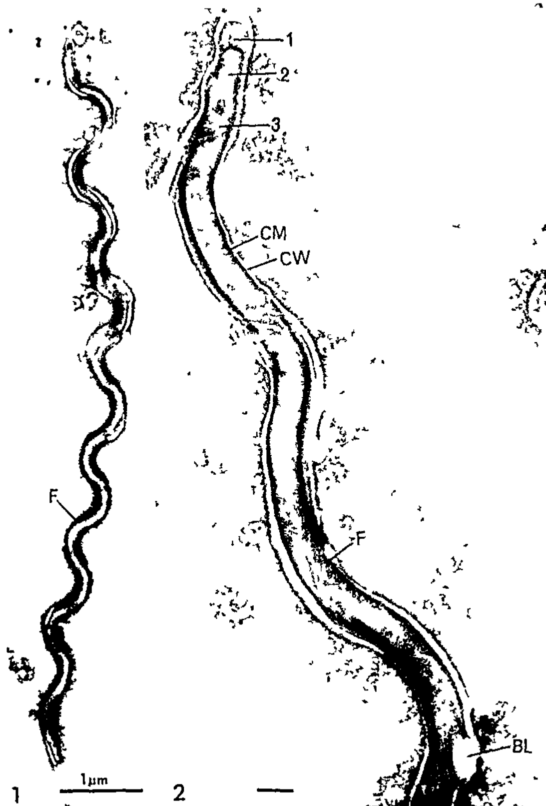
* The SMC solution consisted of 0 03 per cent sucrose, 0 01 M MgCl and 0 01 M CaCl in redistilled water pH was not adjusted but was generally about 5

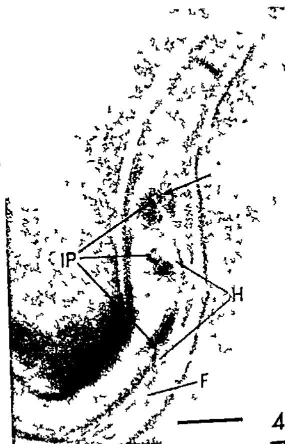
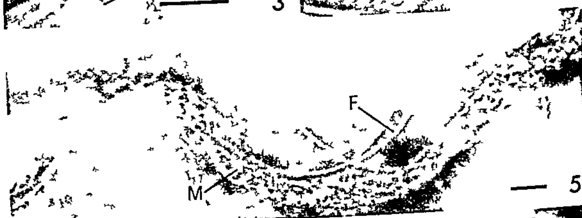
** The purified bacteriolytic enzyme protease I of *Myxobacter* AL 1 was kindly provided by Professor *R S Wolfe* Department of Microbiology University of Illinois Urbana Ill U S A

All illustrations show *Treponema cuniculi* Figs 1 to 12 show organisms negatively stained with 1 per cent ammonium molybdate and Figs *13 to 16* sectioned material obtained from Vestopal embedded blocks The marker on each micrograph represents 100 nm unless otherwise *stated*

Fig 1 Treponeme showing the wavy outline Flagella *(F)* wind around the body of the organism The pointed end at top of the micrograph is particularly well preserved 21 000 ×

Fig 2 The three zones (1–3) (see text) at the end of the organism *are clearly* illustrated Cell wall (CW), cytoplasmic membrane (CM) and flagella (F) are distinct *BL denotes cell* wall bleb 90,000 ×


1
2
3
CM
CW
F
F
BL
1µm
1
2


CM
BL
3

IP
H
F
4

F
M
5

F
M
6

Grain Release Positive Film Type 5302 and suitable fields were photographically enlarged as desired For this paper approximately 350 recordings were studied

RESULTS

Observations on Negatively Stained Material

T cuniculi is a regularly coiled spirochete with pointed ends (Fig 1) The length varies from 8 to 15 μm and the width is about 0 15 μm

At higher magnification the pointed ends are seen to have three zones of varying electron densities (Fig 2) Zone 1, which appears rather electron dense, constitutes the outermost tip and probably consists of cell wall material only A substructure of fine parallel lines is often observed in this part (Fig 3) Zone 2 is somewhat less electron dense and consists of the part of the cytoplasmic body which extends from the end of zone 1 to the region where the flagella are inserted (Figs 2 3 and 4) This part gradually widens till the cytoplasmic body of the organisms has achieved approximately its full width at the site of the insertion of the flagella (Figs 2, 3 and 4) Zone 2 is about 0 16 μm long Zone 3 is the zone around the insertion region

Fig 3 The cross striation at the tip of the treponeme is distinct (arrow) The cytoplasmic membrane (CM) constitutes the borderline between zone 1 and zone 2 (see text) Three flagella (F) with insertion points (IP) lined up in a row One flagellum bends close to the insertion point forming a hook (H) Part of a cell wall bleb (BL) is also seen 160 000 ×

Fig 4 Three flagella (F) with insertion points (IP) lined up in a row Hooks (H) are seen on two of the flagella while the hook is seen end-on on the third (arrow) 160 000 ×

Fig 5 Part of an organism treated on the grid for 10 minutes with 1 per cent sodium deoxycholate The flagella (F) are torn out of the cytoplasm Microtubules (M) are seen with some difficulty in the interior of the cell. This pa[illegible] of the treponeme is somewhat straightened out [illegible]00 ×

Fig 6 Part of an organism ac[illegible]ntally damaged during preparation. Flagella (F[illegible] d microtubules (M) are clearly seen 90 000

of the flagella, and it shows the s[illegible]me electron density as the rest of the cytoplasmic [illegible] of the organism *T cuniculi* has th[illegible] gella inserted at each end General[illegible] flagella are inserted in a row paralle[illegible] long axis of the organisms (Figs 2 3 an[illegible] The mean distance between the in[illegible] points of individual flagella is about 80 nm

Two bundles of flagella, each bundle consisting of three flagella wind around the body of the treponemes (Fig 1) At the middle of the cell the two bundles overlap and the individual flagella of each bundle interdigitate The flagella bend close to their insertion point thus forming a hook (Figs 2 3 and 4)

The cell wall is usually seen in close apposition to the cytoplasmic body, except at the tips of the organism zone 1 as well as in regions showing blebs (Figs 2 and 3) In such regions the outer membrane (cell wall) is found without close contact to the inner structures (cytoplasmic membrane and flagella) Very few cells have shown indication of a slime layer on the outer surface of the cell

Treponemes treated with sodium deoxycholate for a maximum of 10 minutes still retain their wavy appearance though the regularity of the helices is somewhat disturbed This treatment tears a few flagella loose from the cytoplasmic body of the organism (Fig 5)

Thin fibrils with a diameter of 7 nm are revealed in cells treated with sodium deoxycholate (Fig 5) and sometimes these fibrils are also observed in organisms accidentally damaged during preparation for electron microscopy (Fig 6)

Only a few flagella are liberated from the treponemes after treatment for 4 minutes with 0 2 per cent Teepol while the same treatment extended to 10 minutes freed most of the flagella (Fig 7) The treponemes are straightened by the Teepol treatment and the cytoplasmic body of the organisms has a somewhat mottled appearance The loose flagella are seen to consist of a shaft a hook and a basal knob (Fig 7)

Treatment of the treponemes with *Myxobacter* AL-1 protease 1 destroys the organisms, and only flagella, thin fibrils and membranous debris remain (Figs 8 and 9) The structure of the basal knob of the flagella is usually obscured by adhering pieces of membranes A ring-shaped structure can be seen with some difficulty around the basal ends of the flagella (Fig 8) The shaft of free flagella has a diameter of about 17 nm and is covered by a sheath Some micrographs show flagella where the sheath has been removed accidentally (Fig 9) The naked core thus revealed has a diameter of about 10 nm

The thin fibrils are seen in bundles of 6–10 individual fibrils (Figs 9 and 10) At high magnification it can be seen that electron dense staining material has penetrated into the lumen of these filaments, thus demonstrating their tubular nature (Fig 10) Each tubule has a diameter of approximately 7 nm

Organisms of *T cuniculi* divide by transverse fission (Fig 11) New flagella and their insertion organelles are present before the two daughter cells are separated by individual cytoplasmic membranes (Fig 12) Old flagella may still pass across the division site (Figs 11 and 12)

Observations on Sectioned Material

Electron micrographs of ultrathin sections of the skin biopsies show the tissue to be reasonably well preserved (Fig 13) The extracellular space in the *stratum basale* and *stratum spinosum* of the epidermis is somewhat widened, probably as a result of the infection, but many well defined desmosomes are still present The plasma membranes of the cells in the two regions mentioned are quite conspicuous The ribosomes are evenly distributed within the cytoplasm of the cells and the mitochondria do not appear to be swollen and show well defined cristae (Fig 13) A few invading leukocytes are found in the *stratum spinosum* and the basal layer region (Fig 13)

The treponemes are found mainly in the *stratum spinosum* and *stratum basale* layers of the epidermis, and are observed only in the extracellular space (Fig 13) Treponemes have never been observed in the dermis, either between collagen fibres, between fibroblasts, perivascularly, or in the lumen of the small vessels present

Only sinusoidal pieces of treponemes are included in each ultrathin section (Figs 14 and 15) The organisms are surrounded by two tripple-layered membranes with an intermediate layer in between The outer membrane seems to be rather delicate and somewhat difficult to preserve, as it is not visible on all sectioned cells In cross sectioned organisms the flagella are seen situated between the two membranes (Fig 16), *i e* underneath the outer membrane (cell wall), but exterior to the intermediate layer and the cytoplasmic membrane In the cytoplasm close to the cytoplasmic membrane and just underneath the flagella some rather electron dense points with slightly less electron dense centres can be distinguished (Fig 14) These are probably the microtubules revealed in negatively stained organisms after treatment with sodium deoxycholate or *Myxobacter* AL-1 protease 1 The microtubules are also seen in micrographs of cells sectioned obliquely (Fig 14) The nuclear regions of the

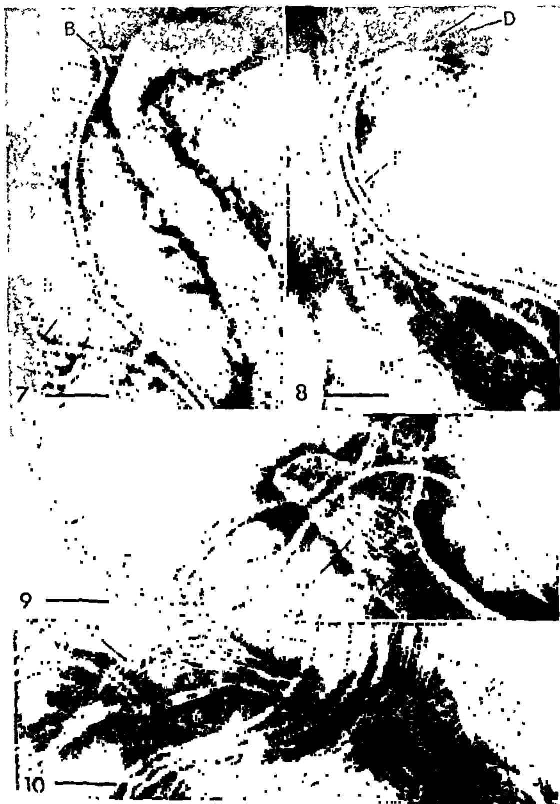
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Fig 7 All flagella have been torn out of this organism which was treated on the grid for 10 minutes with 0 2 per cent Teepol On each of the flagella the substructure of the hook (*H*) is seen to differ from that of the shaft (*S*) The basal knobs (B) are obscured by remnants of membranes 160,000 ×

Fig 8 Part of an organism treated on the grid for 1 minute with 100 μg/ml *Myxobacter* AL 1 protease 1 Only flagella (F), microtubules (M), and membranous debris (D) are left Ring shaped structures are present around the basal ends of two of the flagella (arrows) 160 000 ×

Fig 9 Organism treated as for Fig 8 The sheath on the flagellum (*F*) has been partly removed (arrow) A bundle of microtubules (M) is also seen 160 000 ×

Fig 10 Flagella (F) and microtubules (M) from an organism treated as for Fig 8 The electron dense stain has penetrated into the lumen of the microtubules (arrows) 160 000 ×


B
D
7
8
9
10

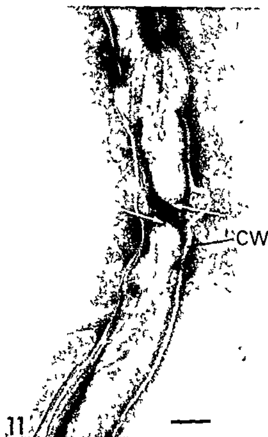




Fig 11 The two daughter cells of a dividing treponeme are separated by their cytoplasmic membranes (arrows), but still enveloped by a common cell wall (CW) 90,000 ×

Fig 12 A treponeme at a very early stage of division where the only sign of division is insertion points (IP) of two new flagella Old flagella (F) pass across the division site, and no constriction is present in the cytoplasmic membrane 90 000 ×

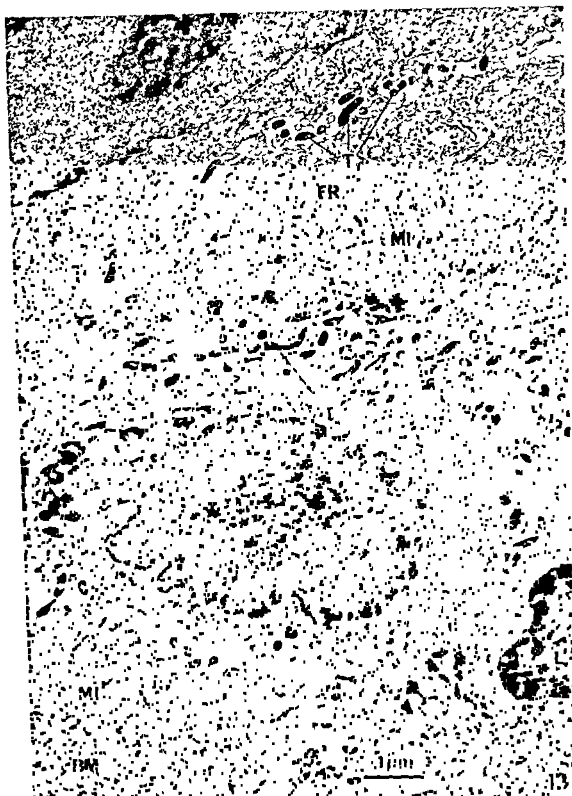
eponemes appear as electron lucent regions the rather dense cytoplasm and thin strands of more electron dense material, probably strands of DNA, are present (Fig 15) Ribosomes are distributed evenly in the cytoplasm Mesosomes have been observed in a few organisms and so have membrane-bound inclusions (Fig 14)

DISCUSSION

The length and width of *T cuniculi* as well as the other observations on negatively stained material of this organism are all identical with corresponding observations on *T pallidum* (12, 14)

The pointed ends of *T cuniculi* are distinguishing features with the three zones each with a definite structure and a characteristic electron density, and with the three flagella inserted in a row subterminally Similar characteristic structures have also been observed at the end regions of negatively

Fig 13 Section of a biopsy from a region of infected skin Treponemes (T) are present in the interspaces between cells of the basal layer and the lower *stratum spinosum* region of the epidermis BM denotes a small part of the basal membrane which separates the epithelium from the underlying connective tissue The epithelial cells do not seem to be much affected by the infection Endoplasmic reticulum (ER) and mitochondria (MI) are seen in the cytoplasm and desmosomes (d) are found between cells An invading leukocyte (L) is present. 15 000 ×


FR
MI
MI
BM
1μm
13

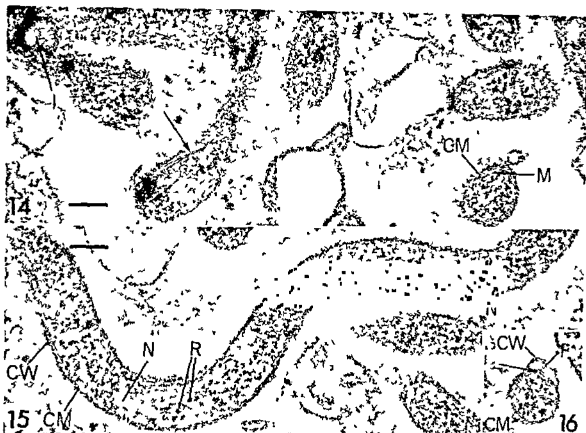


Fig 14 Section of *T cuniculi in situ* between epithelial cells of infected skin Microtubules (M) are seen underneath the three layered cytoplasmic membrane (CM) in a transverse sectioned organism Microtubules can also be distinguished in an obliquely sectioned organism (arrow) A mesosome or a membrane bound inclusion (I) is present in the interior of another cell 90,000 ×

Fig 15 A sinusoidal piece of a treponeme is included in this section Ribosomes (R) and nuclear regions (N) are seen in the interior of the cell CW and CM denote cell wall and cytoplasmic membrane respectively 90,000 ×

Fig 16 A transverse section of a treponeme found between cells of the epithelium of infected skin The ...ella (F) are seen to be situated between the three layered outer part of the cell wall (CW) and intermediate layer (arrow) which is closely apposed to the outer leaflet of the cytoplasmic mem ...ne (CM) 90 000 ×

stained *T pall dum* (12, 14, 18, 20) and on *T pertenue* (10, 19) As the length and width of these three species also are identical it is concluded from this study that *T cuniculi, T pallidum* and *T pertenue* are morphologically indistinguishable by electron mi croscopy of negatively stained organisms

The characteristic pointed ends of *T cuniculi, T pallidum* and *T pertenue* have so far been observed only on these non-cultivable and pathogenic species of *Treponema* Electron microscopy of negatively stained cultivable *Treponema spp* shows the ends of individual organisms of these species to be rather blunt and without the zones of varying elec tron densities The flagella on cells of these species are inserted terminally and more or less haphazardly over the end region (2 3 7, 8, 15 20, 24) The number of flagella per organism varies from species to species but is quite constant in different individuals of each species (7) It thus seems possible on the basis of morphology to distinguish between facultative non pathogenic treponemes with blunt ends and flagella inserted terminally and haphazardly and pathogenic trepo

nemes with distinct pointed ends and three flagella inserted in a row subterminally

T cuniculi and *T pallidum* also appear identical in sections of infected tissues *T cuniculi* appears in great numbers in the extracellular space of the epidermis but has not been found intracellularly or in the dermis Little inflammatory reaction towards the infection has been observed in the biopsies, for example only a few leukocytes have been found As far as we know, up to the present nothing has been published on the location of the treponemes in skin lesions from rabbits with naturally occurring *T cuniculi* infection, either as seen in the light microscope or in the electron microscope

Sectioned material from testes inoculated with *T pallidum* shows the treponemes extracellularly in the interstitial tissue, often *close to the capillaries (12)* The difference in location of the treponemes in rabbit testes inoculated with *T pallidum* (12), as compared to the location of the organisms in skin lesions produced by a natural infection with *T cuniculi* is probably quite insignificant, as the routes of infection for the treponemes in these two cases are so different that the results are incomparable

Work is now in progress in our laboratories to study the difference, if any, in the location of treponemes in skin lesions of primary and secondary human syphilis, and a comparison of these results with those of the present investigation is probably more valid

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SMALL-SIZED HAEMAGGLUTININ OF VESICULAR STOMATITIS VIRUS RELEASED SPONTANEOUSLY AND WITH NONIDET P 40

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Purified vesicular stomatitis virus Indiana serotype disintegrated partly spontaneously in sucrose gradient releasing small haemagglutinin with density of 1 20 g/cm³ Three different structures could be seen in electron micrographs, rosettes fragments of envelope and pieces of nucleoprotein helix with surface structures In polyacrylamide gel electrophoresis viral polypeptides 2, 3 and 4 (numbered in order of increasing mobility) were found Polypeptide 1 was absent or seen only in very small quantities Routine laboratory procedures such as pelleting centrifugation in sucrose, freezing and thawing and also storing at +4°C, increased this disintegration Nonidet P 40 released from viruses structural components from which the haemagglutinating capacity was reactivated after removal of detergent with ether This haemagglutinin had a S value of 65S on the average, banded in CsCl at a density of 1 24 to 1 27 g/cm³ and after purification contained only one viral polypeptide, VP 2 in acrylamide gel electrophoresis This polypeptide is usually considered to be associated with surface projections Electron micrographs revealed aggregates of uniform structures, which in size and shape resemble surface projections

In previous studies the haemagglutinating activity of vesicular stomatitis virus (VSV) has been shown to be associated with the whole infective virion called B particle and also with truncated noninfective T particle, which is only one third of the length of the virion (1, 2, 11) No slowly sedimenting 'soluble' haemagglutinin has been reported to form during VSV or rabies infection (11, 16 21)

Enveloped viruses, on the other hand, are fragile and break up easily In sucrose gradient centrifugation of concentrated VSV preparations rosettes of various sizes and small fringe like structures are found in the top fractions of the gradient (4) The same kind of structures can also be produced from VSV by the widely used Tween 80 and ether treatment (5) However, the haemagglutinating (HA) activity of VSV and rabies is entirely destroyed by this treatment (1, 11) The surface projections of influenza virus can be removed with sodium dodecyl sulphate (SDS) or Tween 20 and again aggregated to form active haemagglutinin (17, 27) It has been recently reported that by treating rabies virus with saponin it is possible to release looped filamentous surface structures with a density of 1 29 g/cm³ and with a haemagglutinating capacity (24) The surface struc-

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SMALL-SIZED HAEMAGGLUTININ OF VESICULAR STOMATITIS VIRUS RELEASED SPONTANEOUSLY AND WITH NONIDET P 40

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tures of rabies or VSV can be released from skeletons with Nonidet P 40 (NP 40), but no haemagglutinating activity was investigated in those reports (7, 10)

In polyacrylamide gel electrophoresis VSV has been reported to contain four viral polypeptides (VP), numbered 1, 2, 3 and 4 in order of increasing mobility The molecular weights of the polypeptides are 230,000, 84,000, 64,000 and 47,000 respectively (15) VP 3 is considered to be polypeptide of nucleoprotein and VP 2 is usually regarded as "spikeprotein" (8, 15)

This report will describe the formation of small sized haemagglutinin through spontaneous disintegration of VSV during laboratory processing and characterize the haemagglutinin formed The preparation of subviral haemagglutinin with Nonidet P 40 will be described in greater detail than in the preliminary report (3), and the properties of this haemagglutinin will be further characterized

MATERIAL AND METHODS

Preparation concentration and purification of haemagglutinin and serological techniques used have been described in detail in the previous reports (1 2) Therefore only a brief outline of these procedures used is described here

Preparation and concentration of virus Vesicular stomatitis virus Indiana serotype was grown in a suspension culture of BHK 21/13S cells maintained in BHK21 medium with 0 4 per cent bovine albumin 10 per cent tryptose phosphate broth and no serum The multiplicity of infection used 0 001 PFU/cell produced more than 95 per cent infective B particles determined in electron microscopy or in sucrose gradients

Extracellular virus was precipitated with polyethylenglycol (PEG) 6000 (20) and the pellet was dissolved in 1/10 volume of 0 05 M Tris HCl buffer (TES) pH 7 4 containing 1 mM of EDTA and 0 15 M NaCl and pelleted again by ultracentrifugation After sonication and removal of debris by low speed centrifugation the resuspended virus was layered on top of 15 to 45 per cent sucrose gradient and sedimented for 2 hours at 20 000 r p m in the Spinco SW 25 rotor The fractions containing whole virions were collected diluted to 5 per cent in sucrose and again pelleted by ultracentrifugation This final pellet was suspended in TES in 1/100 to 1/200 volume of the original virus harvest, and sonicated These virus preparations usually contained about 1 mg/ml of protein according to Lowry s method with albumin as standard protein and were free from contaminating cell or other proteins based on electron microscopical examination and lack of additional polypeptide bands in polyacrylamide gel electrophoresis The concentrated preparations were prepared and used immediately if possible and stored at +4°C when necessary

Estimation of sedimentation coefficients Approximate S values were estimated by centrifuging haemagglutinins in 5 to 20 per cent sucrose gradient with poliovirus as reference (19) For this poliovirus type 1 was grown in LLC MK2 cells Cells were infected with 0 1 PFU/cell of virus after adsorption for 1 hour the cells were washed and BME Diploid medium with 5 μCi/ml of uridine 5 ^{3}H (Radiochemical Centre Amersham spesific activity 29 Ci/mmol) was added Extracellular virus was harvested 24 hours later, pelleted by centrifuging at 85 000 g for 5 hours and purified in CsCl density gradient centrifugation for 44 hours at 35 000 r p m in the Spinco SW 50 1 rotor Purified poliovirus and haemagglutinin were mixed and 0 3 ml of this was layered on top of 5 to 20 w per cent sucrose gradient and centrifuged for 90 minutes at 20 000 r p m in the Spinco SW 50 1 rotor Samples of 5 drops were collected titrated for HA and finally counted in liquid scintillation counter (LKB Wallac 81000 Turku Finland) S values were calculated on the basis of sedimentation velocity of haemagglutinin when compared to poliovirus with known S value of 160 S

Polyacrylamide gel electrophoresis Samples were dialysed overnight at +4°C against 100 to 200 volumes of 0 01 M phosphate buffer pH 7 0 or TES concentrated if necessary by evaporating in dialysis tube and redialysed Sodium dodecyl sulphate (SDS) and 2 mercaptoethanol (2 ME) were added to the samples to a final concentration of 1 per cent for each and the samples were heated for 2 minutes at 100°C After this treatment the mixture was dialysed overnight at room temperature against 100 volumes of 0 01 M phosphate buffer containing 0 1 per cent SDS and 2 ME Acrylamide gel columns (with the length of 55 mm and diameter of 5 mm) were prepared and running of electrophoresis was performed according to Mai el (18)

The samples were mixed with 60 per cent sucrose 4 1 and 0 3 ml of each was layered on polymerized acrylamide gel columns The acrylamide gel contained 5 per cent acrylamide and 0 1 per cent SDS The running buffer 0 1 M sodium phosphate buffer was made in 0 1 per cent in SDS The time of run was about 150 minutes at a current of 8 mA/gel Polypeptide bands were fixed overnight with 20 per cent sulphosalicylic acid and

stained with 0 25 per cent Coomassie blue for 4 hours The excess of stain was washed with 7 per cent acetic acid

A whole virus preparation was included in all subsequent experiments as a control marker for protein mobility The four polypeptides of VSV found corresponded to previous findings when bovine serum albumin was used as reference

Electron microscopy A standard negative staining method was used A drop of each sample was placed on a formvar covered grid and the excess was drained with a filter paper The specimen was allowed to dry and was stained with 2 per cent phosphotungstic acid (PTA) pH 7 0 for 30 seconds The specimens were examined in a Siemens Elmiskop I A at an instrumental magnification of 40 000 using a voltage of 60 kV

RESULTS

Spontaneous Disintegration of Concentrated Vesicular Stomatitis Virus

In the preliminary experiments it was noticed that virus concentrated by PEG precipitation and by ultracentrifugation always contained to some extent, small sized haem agglutinating components, when analysed in 15 to 45 per cent sucrose gradient Because no other haemagglutinating components than B and T particles are known to be produced during VSV infection, this small sized haem agglutinin was regarded as a probable product of the disintegration of virions during the concentration process and the kinetics of this disintegration were studied further

Virus concentrated by precipitation and centrifugation (see Material and methods) was layered on top of 15 to 45 per cent sucrose gradient and centrifuged for 2 hours at 20,000 r p m in the Spinco SW 25 rotor In most cases the upper fractions of the gradient contained some haemagglutinating activity (Fig 1 a), although no visible band could be seen in the gradient tube Fractions containing B particles (black line) were pooled and pelleted by centrifugation for 1 hour at 20 000 r p m in the Spinco 30 rotor, and the pellet was resuspended in TES (in volume of 1/100 to 1/200 of the original) The effect of storing was tested by keeping viruses at + 4°C for different times Recentrifugation in 15 to 45 per cent sucrose gradient after storing for 2 days resulted in the release of small sized haemagglutinin in an equal titre as whole virus haemagglutinin

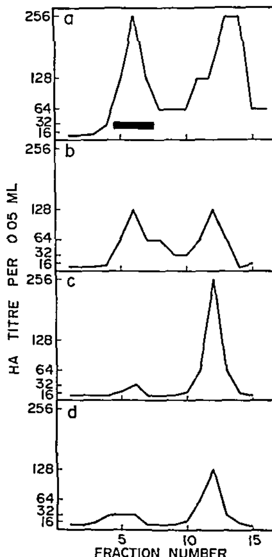


Fig 1 The release of spontaneous small sized haemagglutinin in sucrose gradient centrifugation after storage for different times By precipitation and centrifugations concentrated virions were layered on top of 15 to 45 per cent sucrose gradient and centrifuged for 2 hours at 20 000 r p m in the Spinco SW 25 rotor (a) Fractions containing B particles (Black line) were pooled and virions pelleted by centrifuging for 1 hr at 20 000 r p m in the Spinco 30 rotor The resuspended pellet was stored at +4°C and a part of it was recentrifuged after one day (b), 2 weeks (c), and after 6 weeks (d)

TABLE 1 *Preparation of Small Sized Haemagglutinin with Different Concentrations of Nonidet P 40**

Final concentration of Nonidet P 40 (%)	HA titres in water phase per 0 05 ml
PBS	8
0 01	8
0 05	8
0 10	16
0 15	256
0 20	256
0 25	1024
0 30	< 2
0 35	< 2
0 40	< 2
Control**	2048

* Purified and concentrated VSV preparation contained 1 2 mg/ml protein 0 2 ml of this virus suspension was mixed with 0 05 ml of Nonidet P40 to make the final concentration listed above After incubating for 10 minutes at room temperature the mixture was shaken with an equal volume of ether and centrifuged for 5 minutes at 1,500 r p m and the water phase was collected

** Control was 0 2 ml of the same virus suspension with 0 05 ml of PBS

(Table 1) The optimal final concentration of NP 40 with the virus preparation used, was usually 0 2 per cent The haemagglutination titres varied greatly, from an eight-fold increase to an eight fold decrease compared with the original Optical density of water phase increased in relation to the detergent concentration Replacing NP 40 with PBS and treating the preparation identically in other respects resulted in very low haemagglutination titres The theoretical possibility that the haemagglutinating capacity of water phase was due to the straight influence of possible residual ether left in the water phase, was excluded by treating erythrocytes with various concentrations of ether and the red cells showed no nonspecific agglutination

Water phase seemed also to be moderately free of NP 40, since no haemolysis took place in haemagglutination titration of water phase, not even in the lowest dilution of 1 2 Also when measuring optical density the high additional absorbance at wavelength 280 nm due to Nonidet P 40 (23) was removed after ether treatment

Sucrose and CsCl Centrifugations of NP 40 Released Haemagglutinin

Haemagglutinin of water phase prepared with Nonidet P 40 as described above, was layered on top of 5 to 25 w per cent sucrose gradient without detergent, centrifuged for 90 minutes at 35,000 r p m in the Spinco

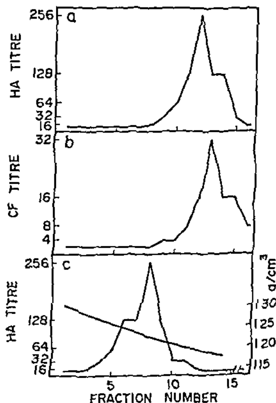


Fig 5 Centrifugation analyses of haemagglutinin after treatment of virions with 0 2 per cent Nonidet P 40 for 10 minutes at 22°C and removal of detergent with ether After sedimentation centrifugation in 5 to 25 per cent sucrose gradient at 35,000 r p m for 90 minutes in the Spinco SW 40 rotor, both the HA activity (a) and the CF activity (b) were found in one peak Haemagglutinin of water phase banded in one peak after centrifuging for 16 hours at 32 500 r p m in the Spinco SW 39 rotor in preformed CsCl gradient with densities of 1 13 to 1 30 g/cm³ The peak of haemagglutinin corresponded to a density of 1 24 g/cm³

SW 40 rotor and fractions were collected. The haemagglutinating activity was found in one peak. The major CF activity was found in the same peak although some CF activity also remained at the top (Fig. 5 a and b). The approximate S value of this haemagglutinin was 40 to 90S, on the average 65S.

After centrifugation of haemagglutinin in a linear, preformed CsCl gradient with a density of 1.13 to 1.30 g/cm³ for 16 hours at 35,000 r.p.m. in the Spinco SW 39 rotor the haemagglutinin was found again in one peak banding at a density of 1.24 g/cm³ (Fig. 5 c). In some experiments the density was somewhat higher, up to 1.27 g/cm³.

Polyacrylamide Gel Electrophoresis of NP 40 Released Haemagglutinin

The crude water phase preparations contained from 2 to 4 viral polypeptides depending on the concentration of Nonidet P 40. The interphase contained all viral components in great concentrations, as expected, in the ether phase no particular viral polypeptide was found with any regularity.

After purification of NP 40 water phase haemagglutinin according to sedimentation properties in 5 to 25 w per cent sucrose gradient viral polypeptides 2 and 3 were constantly found (Fig. 6). To obtain increased purity fractions from the sucrose gradient were pooled, concentrated and dialysed against TES and centrifuged in the CsCl gradient, as above. Fractions containing haemagglutinin were pooled and diluted to 5 ml of TES and pelleted by centrifuging for 16 hours at 40 000 r.p.m. in the Spinco 50 rotor. After pouring out the supernatant, a drop of distilled water was pipetted to the bottom of the tube, resuspended and a small amount mounted on the grid for electron microscopy. Then 0.5 ml of TES was added and the bottom thoroughly rinsed and the preparation further processed for polyacrylamide gel electrophoresis. In this preparation only one polypeptide was found moving with equal electrophoretic mobility than viral polypeptide 2 in control virus preparation.

Fig. 6. Polyacrylamide gel electrophoresis of small sized haemagglutinin of VSV released with Nonidet P 40, purified first according to sedimentation properties (in the middle) and then in a density gradient. Fractions from the density gradient containing haemagglutinin were pooled and concentrated by pelleting for 16 hours at 40 000 r.p.m. in the Spinco 50 rotor. Pellet was first resuspended in one drop of distilled water and grids were prepared for electron microscopy (See Fig. 7). 0.5 ml of TES was added and resuspended pellet further processed for acrylamide gel electrophoresis. The purified haemagglutinin is on the right and control virus on the left. VP 1 to 4: viral polypeptides in order of increasing mobility.

Electron Microscopy of NP 40 Released Haemagglutinin

In electron micrographs the only material found in a sample of pelleted haemagglutinin, purified as above according to sedimentation properties and density, was a netlike structure of aggregated small subunits (Fig. 7). The size and shape of these structures resembled the surface projections of whole virions. No structures resembling those of spontaneously disintegrated virus, such as rosettes, fragments of envelope or nucleoprotein helix, could be seen. The subunits of NP 40 released haemagglutinin were joined to



Fig 7 Electron micrographs of the same with Non det P 40 released haemagglutinin as shown in Fig 6 showing large aggregates of uniform structures Negatively stained with 2 per cent PTA pH 7 0 The bar represents 100 nm

each other by end to end or by end to side joints forming the netlike image of large aggregates

DISCUSSION

The haemagglutinating activity of bullet shaped viruses has been reported to be associated with the whole virions only or with the defective T particles of VSV (1 2 11) Haemagglutinating activity of enveloped viruses is usually associated with the envelope and particularly with the surface projections (17) On the other hand enveloped virions are labile and disintegrate during normal laboratory procedures It seems reasonable to assume that various particles with surface structures and with HA activity could be formed during viral disintegration Bullet shaped viruses are sensitive for centrifugations and for rapid changes in osmotic pressure (9, 13) In our previous and present studies concentrated VSV preparations contained in addition to B and T particles three different kind of structures with surface projections rosettes fragments of nucleoprotein helix with envelope and pieces of envelope which were not rolled up to form a rosette All these structures banded at a density of 1 20 g/cm³ suggesting the presence of envelope lipids With Newcastle disease virus phospholipids can form a filament where surface projections might be attached to form active haemagglutinin (14) In the case of VSV it remains unclear whether rosettes and unrolled pieces of envelope are formed from one structural protein of VSV primarily from VP 2 believed to be spike protein (8) and the sub units are connected to each other by phospholipid filaments or whether VP 4 is also present in the formation of these structures VP 1 the role of which is obscure is almost entirely absent from spontaneously disintegrated haemagglutinin VP 3 believed to be nucleoprotein (8 15) was found surprisingly often in the preparations containing small sized haemagglutinin released spontaneously or by NP 40 It has been suggested that viral spikes of VSV might be attached to layers under envelope or even to nucleoprotein helix (6)

Examinations of the kinetics of this spontaneous disintegration confirmed previous results of the lability of VSV during routine laboratory procedures Some stabilizing substance as urea by rubella (26) might be useful while handling haemagglutinin of VSV

With most detergents the difficulty was a rapid loss of all HA activity In our preliminary screening tests however no attempt was made to remove the detergent which is necessary by influenza virus for instance (17 27) Tween 80 might have a straight influence on receptor sites of VSV or goose erythrocytes because HA activity is lost even in very low concentrations probably insufficient to break viruses up NP 40 was known

to release surface structures of VSV (7) and because it was easy to remove NP 40 with ether totally or almost totally, this detergent was chosen. After ether treatment haemagglutinating activity could be recovered in the water phase. The precise optimal concentrations could not be predicted beforehand, but it was necessary to test this with small quantities. This distinct area of proper preparation could be a matter of micelle formation and solubilization of proteins. The splitting of *Chloroplast lamellae* with NP 40 is known to take place in 4 distinct steps, depending on the concentration of detergent (25).

The maximal rise in HA titres was four- to eight fold, but the final titre could also be less, possibly due to several factors. NP 40 has been reported to certain extent to adhere to membrane proteins (12), after NP 40 treatments the preparations of Inkoo virus, containing small haemagglutinin, have a slightly different pH optimum for HA (22), it might also be due to aggregation of HA subunits.

The NP 40 haemagglutinin was noticed to be small and sediment slowly as a rather broad band corresponding to S values of 40 to 90S, 65S on the average. The other properties of this haemagglutinin included banding at a density of 1.24 to 1.27 g/cm^3, and only one viral polypeptide, VP 2, usually believed to be the polypeptide corresponding glycoprotein of spikes (8).

The electron micrographs revealed uniform structures corresponding to the size and form of aggregated projections. These kinds of structures have never been seen in other VSV preparations. The preparations seemed to be free from other envelope structures. The looped filaments with haemagglutinating activity, released from rabies virus by saponin treatment look rather alike (24).

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THE EFFECTS OF DETERGENT-TREATMENT ON THE MORPHOLOGY OF AUSTRALIA ANTIGEN POSITIVE PARTICLES

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On treatment of a serum positive for Australia antigen with increasing concentrations of the detergent Tween 80, an advancing disintegration of the Australia antigen particles can be demonstrated It is noteworthy that the Australia antigen tubules after having passed a stage of periodical constrictions of approximately 20 nm eventually disappear completely, while the 20 nm spherical Australia antigen particles remain The Dane particles (42 nm) showed increasing destruction of their outer Australia antigen containing membrane finally releasing a 27 nm particle with "capsomere like structures on its surface At the highest detergent concentration utilized (0 5 per cent), no particles could be demonstrated in the specimens The 27 nm particles were aggregated by sera containing no Australia antibodies or antigen, drawn from patients who had previously undergone virus B hepatitis It is concluded that the 20 nm Australia antigen particles may be a secondary phenomenon produced by a detachment mechanism from the tubules only the latter being actually produced in connection with virus replication

Since *Blumberg et al* demonstrated Australia antigen (Au/SH antigen HAA) in 1964–65 (3), evidence has accumulated that this represents a specific serological marker for virus B ('serum –) hepatitis (6, 8) The exact nature and origin of the antigen has not yet been established, however, and there have been no convincing reports of the cultivation of hepatitis virus *in vitro*, or in laboratory animals

Electron microscopic studies (2, 4) have shown that the Australia antigen-specificities are associated with three different types of particles

1 The 'classical' Australia antigen particle This is spherical and has a diameter of approximately 20 nm

2 The tubular particle This also has a diameter of approximately 20 nm and may be several hundred nm long Constrictions resulting in a spherical appearance have recently been described (5)

3 The so-called "Dane particle' (4) has a diameter of 42 nm and, in the electron microscope, it has the appearance of a corona-, or paramyxo-virus It is found only in some of the sera positive for Australia antigen, and there is considerable variation in the number of Dane particles in the different positive sera (4)

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Several hypotheses have been proposed for the origin of Australia antigen, and for the relationships between the different types of particles The most generally accepted point of view, at the present time, seems to be that Australia antigen represents excess viral protein produced in the liver cells during the virus replication cycle General agreement also seems to exist that, if any of the known particle forms represents the hepatitis virion it is probably the Dane particle (10)

The current hypotheses offer however no explanation for the production of several different types of particles, such as the three forms described above

This publication reports how different concentrations of the detergent Tween 80 influence the morphology of the various particle types On the basis of the observations a possible explanation for the heterogeneity of the Australia antigen particles is presented The discovery of a new antigen antibody system, previously reported by *Almeida et al* (1), is also confirmed in this work

MATERIALS AND METHODS

Reagents

I Sera

A commercial Australia antigen positive serum (Spectra Biologicals lot no 214 2) was used in all experiments The following antisera were employed in the investigations

1 A human antiserum against Australia antigen (Spectra Biologicals lot no 145)

2 A serum containing antibodies against the 27 nm particle This was a gift from dr *June D Almeida* Royal Postgraduate School of Medicine London to whom we are indebted

3 The serum of a patient (A T), who had undergone an Australia antigen positive hepatitis 6 months prior to the collection of the serum This serum is negative for Australia antigen and antibodies by counter electrophoresis and a modified gel diffusion test (9)

II Detergent

'Tween 80 Fluka, polyoxyethylene sorbitan monooleate was employed in these experiments

Detergent Treatment

0 3 ml antigen containing serum was diluted to 3 ml in PBS (phosphate buffered saline) pH 7 and centrifuged for 1 hour in a Sorvall RC2 B centrifuge, with rotor SS 34 at 18000 rpm (20000 g) The supernatant was discarded, and the pellet resuspended in 0 05 0 1 0 25 and 0 5 per cent Tween 80 in PBS The suspension was incubated for 30 minutes at room temperature before being centrifuged for 1 hour at 20000 g The pellet was then utilized as antigen in immunoelectron microscopy

Preparation for Immuno Electron Microscopy

The pellet of the detergent treated antigen was resuspended in 0 25 ml PBS and 0 1 ml antiserum was added The suspension was mixed carefully and incubated overnight at +4° C The following morning the suspension was diluted to 3 ml with PBS and centrifuged 1 hour at 20000 g The supernatant was discarded and the pellet was submitted to electron microscopy after staining

Electron Microscopy

The pellet of the detergent treated antigen after preincubation with antibody, was resuspended in 0 05 ml distilled water One drop of this suspension was mixed with one drop of 3 per cent phosphotungstic acid adjusted to pH 6 with KOH and one drop of this mixture then placed on a 200 mesh carbonformvar coated grid Surplus fluid was removed with filter paper and the grid examined in a JEM electron microscope at a magnification of 60000 ×

RESULTS

The commercial serum (Spectra Biologicals) was chosen as a source of Australia antigen for this investigation, as it was found to contain by electronmicroscopy all three kinds of antigen particles In addition to the 20 nm particles, there was a considerable number of tubules and intact Dane particles (Figure 1) The latter were penetrated by stain to varying degrees and thus clearly demonstrated an outer membrane and an inner core', the so called 27 nm particle

After detergent treatment the electron microscopic distribution of the various particles changed The alteration gradually became more evident as the concentration of Tween 80 was increased from 0 05 per cent to 0 5 per cent At the lower detergent concentration a partial rupture of the outer membrane of the Dane particle was observed with the inner 27 nm particle being released

in some cases At the same concentration of detergent, the small 20 nm particles and the tubules were penetrated by stain to a certain degree, demonstrating a distinct outer shell and an electron transparent central area (Fig 2 a and 2 b) At this lower detergent concentration, however, a considerable number of Dane particles remained

When the detergent concentration was increased to 0 1 per cent a larger number of disrupted Dane particles was seen Some of the inner components (27 nm particles) were observed as free particles with the outer membranes as rims beside them The tubules showed a distinctive narrowing (Figures 3 a and 3 b) The micrographs revealed periodical constrictions along the whole length of the tubules, and they gave the impression of being composed of 20 nm spherical subunits (Figures 3 a and 3 b) In addition, a large number of the micrographs, irrespective of detergent treatment, indicated spherical 20 nm particles at one or both ends of the tubules (Figure 4)

When the detergent concentration was increased to 0 25 per cent, no tubules were seen The micrographs showed free 27 nm particles and 20 nm particles, strongly penetrated by stain

At the highest concentration of Tween utilized (0 5 per cent) no particles of any kind were observed, as no aggregates were present

Micrographs of the 27 nm particles indicated that they were surrounded by an outer shell composed of repeating subunits with a diameter of approximately 4 nm (Figure 5) The figure gives no information about the interior of the particle, since this was penetrated by stain

A specific "anti core" antiserum received from Dr *June D Almeida*, aggregated 27 nm particles in our Tween treated (0 1 per cent) Australia antigen serum (Figure 6) The same effect was attained by mixing our Tween treated antigen serum with the serum of a selected patient (A T) This patient suffered from jaundice and was diagnosed as Australia antigen positive 6 months before the serum used in this investigation was drawn This serum is negative for Australia antigen and its homologous antibodies

DISCUSSION

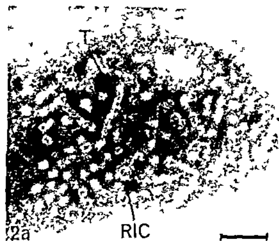
Treatment with Tween 80 under certain conditions seems to lead to the removal of 20 nm spherical particles from the tubular Australia antigen particles This raises the question of whether this detachment is a process which also occurs under natural conditions This may well be the case, and this mechanism offers an explanation for the large variations in the lengths of the tubules from the same specimen, and also for the variations in the relative amounts of the two particle types from specimen to specimen However, it is difficult, at the present time, to give any probable explanation as to the factors involved in 'triggering" such a detachment in vivo

Tubular particles as a result of intracellular virus activity is not a peculiarity of virus B hepatitis Corresponding particles have recently been demonstrated in certain togavirus infections of infant mouse brains (7) They are found at the site of replication in the cytoplasma The authors state that the particles usually were similar to virus particles in diameter' This is also the case for the Australia antigen tubules, if it is assumed that the 27 nm core of the Dane particle represents the virion of virus B Whether the tubules of togaviruses also differ from the virions in their antigenic composition has not yet been investigated

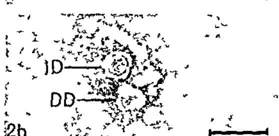
Our studies confirm the recent report of *Almeida et al* (1) that, after Tween 80 treatment, a new antigenic specificity is exposed for the Dane particle This specificity is associated with the 27 nm particle which is released after disrupting the Au antigen positive protein shell of the 42 nm particles The 27 nm particles are penetrated by stain to varying degrees, and have the morphological appearance of a picorna virus Sera from patients who have previously undergone a virus B hepatitis aggregate the 27 nm par-



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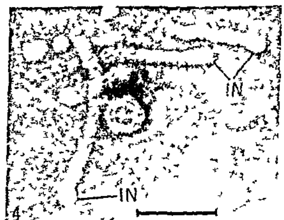
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C
3b



IN
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4



ticles, even if they are negative for Australia antibodies and do not, therefore, aggregate the intact Dane particles In some of the micrographs, in the 27 nm particle, subunits resembling capsomeres were observed

Almeida et al have used 0 5 per cent Tween 80 in their experiments When em ploying the same detergent concentration and the same experimental conditions, none of the types of Australia antigen particles were observed in the specimens No obvious explanation of this discrepancy is apparent but the lipid concentration in sera can var considerably, and thus may affect the actio of the detergent on the Au particles

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Unless otherwise stated the bar on each mic graph represents 0 1 μm All preparations w negatively stained with 3 per cent phosphotung acid The following abbreviations are used D Dane particle T = tubular form SP = sphe particle ID = intact Dane particle DD = rupted Dane particle RIC = released inner C – constriction IN indication of 20 nm s cal particle S = subunit

Fig 1 Au antigen antibody complex. All types of Au-antigen particles are intact Magnifica tion 150000 ×

Fig 2 a Au antigen positive serum treated with 0 05 per cent Tween 80 and reacted with anti body 20 nm spherical particles and tubular forms show a distinct outer shell and a central electron transparent area The 42 nm particles are partly ruptured and the inner 27 nm spherical component released Magnification 120000 ×

Fig 2 b As in Fig 2 a Intact and disrupted Dane particles in the same preparation Magnification 150000 ×

Fig 3 a Au-antigen positive serum treated with 0 1 per cent Tween 80 and reacted with post hepatitic serum Tubular forms with periodical constrictions along the whole length. Intact and disrupted Dane particles and free 27 nm particles Magnification 180000 ×

Fig 3 b As in Fig 3 a Tubular forms with con strictions Magnification 180000 ×

Fig 4 Au antigen positive serum Tubular forms with indications of 20 nm spherical particles at one end Magn fication 200000 ×

Fig 5 As in Fig 3 a Released 27 nm particles with an outer shell composed of subunits ca 40 Angstrom in diameter Magn fication 270000 ×



Fig 6 Au antigen positive seru 1 per cent Tween 80 and specific antiserum against 27 nm particles Release and aggregation of the 27 nm particles. Magnification 180000 ×

In conclusion, the following working hypotheses may prove valuable in further studies

The spherical 20 nm Australia antigen particles are produced by detachment from the tubules and may thus represent a second ary phenomenon The tubules are produced by the liver cells under the influence of hepa titis virus

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GLUTAMATE DEHYDROGENASES IN *NEISSERIA MENINGITIDIS*

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In cell free extracts from *Neisseria meningitidis* 2 species of glutamate dehydrogenase are found by agar gel electrophoresis one NAD linked and the other NADP linked The activity of the NAD linked enzyme is decreased when glucose is added to the medium while the activity of the NADP linked one is increased by the same conditions This observation is consistent with the hypothesis that NAD linked GDH mainly has a degradative function while the NADP linked one is active in the biosynthesis of glutamate

Glutamate dehydrogenase (GDH) from animals and higher plants usually utilizes both NAD and NADP as coenzymes (18) In contrast the GDH in microorganisms is specific for either NAD or NADP (17) GDH in *Mycoplasma laidlawii* makes an exception, as the same enzyme can function with NAD as well as NADP (20) In yeast and several fungi 2 different GDHs are found, one coupled to NAD and the other one to NADP, whereas most bacteria contain only one GDH, either the NAD or the NADP linked one (17) However both NAD- and NADP-linked GDHs are found in the autotrophic bacteria *Thiobacillus novellus* (15) and *Hydrogenomonas eutropha* (5) It appears that the NAD linked GDH is most active during growth in glutamate containing media, while the NADP-linked enzyme dominates when the organisms are grown under autotrophic conditions. The NAD linked enzyme is assumed to have a degradative function, while the NADP linked one is responsible for the synthesis of glutamate from α keto glutarate and ammonia (5, 15, 18)

Jyssum & Borchgrevink (9) demonstrated that in *Neisseria meningitidis* GDH is coupled to both NAD and NADP The NAD linked activity was stimulated in cells grown on a medium supplemented with glutamate, and on a basal medium with NH_4^+ as the sole nitrogen source, while the NADP linked activity remained unaffected by these conditions It was suggested that the NAD linked activity might be responsible for the oxidative deamination of glutamate

The present findings show that *N meningitidis* contains 2 species of GDH, one NAD linked and one NADP linked (l glutamate NAD oxidoreductase (deaminating), E C 1 4 1 2, and l glutamate NADP oxidoreductase (deaminating), E C 1 4 1 4) (1) Some aspects on the regulation of these enzymes are studied for use in comparative investigations among different *Neisseria* species

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MATERIALS AND METHODS

Strains The strains of *N meningitidis* used are listed in Table 1 Strain No 15 was obtained from dr *B W Catlin* Marquette University School of Medicine Milwaukee Wisconsin U S A, and

TABLE 1 *Strains of N meningitidis Used in the Experiments*

Strain	Serogroup	Source	Reference
M 1	B	Cerebrospinal fluid	6
M 5	C	Cerebrospinal fluid	6
M 6	B	Cerebrospinal fluid	6
B 8152/66	A	Cerebrospinal fluid	13
Ne 15	B	Cerebrospinal fluid	2
ATCC 13113	D	Cerebrospinal fluid	
P 5	A	Cerebrospinal fluid	3
P 17	Y	Cerebrospinal fluid	
P 19	X	Cerebrospinal fluid	
P 22	C	Cerebrospinal fluid	
1335	Y	Nasopharynx	4
1875	Z	Nasopharynx	4

strain ATCC 13113 from dr *Alice Reyn*, Statens Seruminstitut, Copenhagen Denmark

Media Blood agar was used as routine medium for preparing cell free extracts for electrophoresis and screening of GDH activity Extracts for the examination of the properties of GDH and for the examinations of the influence of glucose were prepared from cells grown on KC medium (14) This medium contained per litre K_2HPO_4 7 g, KH_2PO_4 2 g, NH_4Cl 1 g, Na_2SO_4 0 05 g, $Na_2S_2O_3$ 0 05 g, $MgCl_2$ 0 1 g, $CaCl_2$ 0 05 g, $MnCl_2$ 0 001 g, l glutamic acid 1 g, l arginine HCl 0 3 g, l cysteine HCl 0 01 g glycerol 50 per cent 1 ml, K lactate 21 8 per cent 25 ml. To obtain a solid medium 15 g agar was added per litre

Preparation of extracts The cells were harvested from the surface of solid media after incubation for 18 hours at 37° C in humid atmosphere with increased CO_2 content After being washed once in saline and resuspended in 0 05 M Tris/HCl buffer at pH 7 4, the cells were disrupted by 2 treatments of 90 seconds each in a MSE ultrasonic disintegrator During treatment the vessel was cooled in an ice salt water bath Cell debris was removed by centrifugation at 10000 × g for 30 minutes All preparative manipulations were carried out at ice water temperature (12) The extracts were stored at 20° C

Assay of GDH activity The procedure followed that described by *Jyssum & Borchgrevink* (9) The reaction mixture for the reduction of NAD and NADP contained KCN 1 5 μmoles nicotinamide 1 2 μmoles sodium l glutamate 10 μmoles NAD or NADP 0 5 μmoles extract dilution 0 1 ml and Tris/HCl buffer at the pH indicated 100 μmoles The total volume was 2 6 ml The reaction mixture for the reverse reaction contained reduced coenzyme 0 25 μmoles and NH_4Cl 60 μmoles and α ketoglutarate 10 μmoles as substrates Reduction and oxidation of coenzymes were followed at 340 nm in a Hilger Gilford Reaction Kinetics recording spectrophotometer

Agar gel electrophoresis Electrophoresis was carried out on object slides in a LKB electrophoresis equipment. The agar gel contained 1 2 per cent Difco Special Agar Noble in sodium barbital buffer pH 8 6, ionic strength 0 025 The electrophoresis was run for 90 minutes at 250 V, after which the enzymes were localized by covering the gel with the following mixture Difco Special Agar Noble 0 67 per cent in sodium barbital buffer pH 8 4 ionic strength 0 1, l glutamate 5 mM, NAD or NADP (or both) 0 05 per cent, nitro blue tetrazolium 0 015 per cent, phenazine methosulphate 0 0015 per cent (19) GDH activity appeared as blue bands

Protein determination Protein was determined with the Folin phenol reagent (16) with bovine serum albumin as standard

Chemicals α ketoglutarate NAD NADH, NADP, NADPH, phenazine methosulphate, and cyclic AMP were obtained from Sigma Chemical Company, St Louis Minnesota USA, sodium l glutamate from BDH Chemicals Ltd, Poole England, and nitro blue tetrazolium from Koch Light Laboratories Ltd Colnbrook Buckinghamshire England

RESULTS

GDH activity was determined in the sediments and supernatants after centrifugation at 10000 and 100000 × g Almost no activity was found in the sediments while the 100000 × g supernatant contained strong GDH activity

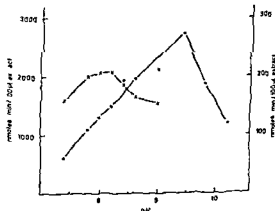


Fig 1 pH optima for meningococcal glutamate dehydrogenase Left ordinate Oxidation of *NADPH* (x——x) Right ordinate Oxidation of *NADH* (●——●)

TABLE 2 *Glutamate Dehydrogenase Activity in Different Strains of N meningitidis*

Strain	Coenzyme NAD	NADH	NADP	NADPH
M 1	8 9	108 7	106 5	1330 4
M 5	13 7	34 9	63 6	564 0
M 6	10 5	70 5	108 9	733 1
B 8152/66	15 1	65 7	103 2	1295 7
Ne 15	9 4	92 7	58 5	777 8
ATCC 13113	2 0	3 0	10 2	83 7
P 5	5 2	24 0	60 0	629 6
P 17	8 1	74 2	111 3	1655 8
P 19	9 2	34 4	51 8	627 2
P 22	9 6	28 3	134 3	1204 0
1335	5 5	22 8	47 5	457 9
1875	15 3	117 7	82 1	814 1

Activities are expressed as nmoles substrate converted per min per mg protein

pH optimum The activity of the GDH at different pH values is shown in Figure 1 At pH > 9 0 glycin/NaOH buffer was used The NAD-linked activity had its pH optimum at 9 4, while for the NADP linked activity the optimal pH values was 8 0–8 2 In the following experiments Tris/HCl buffer at pH 8 0 was used when NADP/NADPH was the coenzyme, and pH 9 0 in the reactions with NAD/NADH

GDH activity in different strains of N meningitidis Extracts from different strains of *N meningitidis* were examined for GDH activity (Table 2) The extracts were made from blood agar grown cells, and the activities were measured at pH 8 0 No great differences between the strains were found, except that strain ATCC 13113 had a rather low GDH activity

Temperature sensitivity Extracts from strain M 6 was placed in a water bath for 3 minutes at the temperature indicated, chilled in an ice water bath, and then examined for GDH activity The NAD-linked activity was rapidly destroyed at 60°C, but the NADP linked activity was somewhat more resistant, being unaffected by 3 minutes at this temperature (Figure 2)

Effect of glucose on GDH activity Extracts were made from strain M 6 grown on KC-medium supplemented with glucose at different concentrations The NAD linked activity decreased with increasing glucose concentration in the medium, whereas the NADP-linked activity increased (Figure 3) The strain B 8152/66 was used in an attempt to find out whether this effect was exerted by the unchanged glucose molecule Strain B 8152/66 lacks the glucokinase (13), and is accordingly not able to metabolize glucose Extracts from this strain were pre-

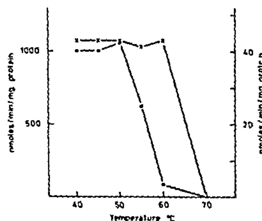


Fig 2 Temperature inactivation curves for meningococcal glutamate dehydrogenase Left ordinate Oxidation of NADPH x——x Right ordinate Oxidation of NADH ●——●

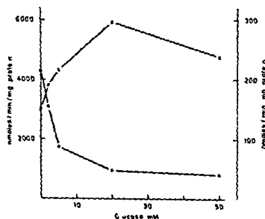


Fig 3 Effect of glucose upon the activity of meningococcal glutamate dehydrogenase Left ordinate Oxidation of NADPH (x——x) Right ordinate Oxidation of NADH (●——●)

TABLE 3 *Glutamate Dehydrogenase Activities in Strain M 6 and B 8152/66 Grown on Solid and Fluid KC medium in the Absence and Presence of G 6 P*

	Medium	Addition	NAD	Coenzyme NADH	NADP	NADPH
Strain M 6	Solid KC	None	29 1	196 1	200 0	2034 8
		G 6 P	25 4	167 4	130 9	1743 5
	Fluid KC	None	44 6	249 6	175 6	1670 6
		G 6 P	15 4	200 0	149 3	1476 5
Strain B 8152/66	Solid KC	None	47 2	218 3	101 7	1891 3
		G 6 P	43 7	174 3	87 2	1452 2
	Fluid KC	None	49 2	349 3	249 3	2064 7
		G 6 P	51 5	323 9	175 6	1964 7

G 6 P was added at a concentration of 10 mM Activities are expressed as nmoles substrate converted per min per mg protein

pared from the same media as described above In these extracts there was no change in the GDH activities as the concentration of glucose was increased

To circumvent the defect glucokinase the meningococci were grown on KC medium supplemented with 10 mM glucose 6 phosphate (G 6 P) Strain M 6 has previously been shown to metabolize this substance (10) Extracts were made from the strains M 6 and B 8152/66 grown on KC medium with and without G 6 P In these strains both the NAD and the NADP linked GDH activity was slightly less in cells grown in the presence of G 6 P The same decrease in activity was noted in cells grown for 4 hours in liquid KC medium with the same composition as the solid one (Table 3)

Cyclic AMP had no effect on the GDH activity at a glucose concentration of 5 mM

Agar gel electrophoresis The GDH activity was clearly separated in 2 bands both of them migrating towards the anode and the NAD linked one covering the greatest distance (Figure 4) No cross reactivity could be detected by these means only one band was coloured with either of the coenzymes All the strains listed under Materials and Methods were tested Except for the strain ATCC 13113 the distance of migration for the 2 enzymes were identical with those of strain M 6 In strain ATCC 13113 no band

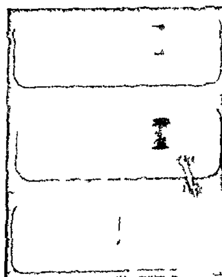


Fig 4 Agar gel electrophoresis of meningococcal glutamate dehydrogenase Top NAD and NADP linked activities centre NADP linked activity bottom NAD linked activity The anode is to the right

corresponding to NAD linked activity could be found although extract from this strain possessed NAD linked GDH activity (Table 2) Possibly the concentration of enzyme was too low to be detected in the agar gel NADP linked activity was demonstrated in this strain and the corresponding band migrated exactly as far as that from strain M 6

DISCUSSION

It is clear from Figure 4 that *N meningitidis* contains 2 species of glutamate dehydrogenase, one NAD linked, and one NADP linked. Both enzymes are found in the 100000 × g supernatant, in contrast to those of *H eutropha*, which were found both in the supernatant and the sediment after centrifugation at 39000 × g (5).

The 2 enzymes differ in their sensitivity to high temperatures, and they have different pH optima. In *T novellus* (15) NAD linked GDH was rapidly inactivated at 65° C, and the NADP-linked one at 75° C. This is in accordance with the findings in *N meningitidis*. The pH optima of the *T novellus* enzymes for the oxidation of NADH and NADPH were 9.0 and 8.0 respectively, results that are quite similar to those obtained in the present experiments.

The NAD linked GDH was less active in meningococci grown on KC medium supplemented with glucose, and the activity of the NADP linked enzyme was increased under these conditions. This observation is consistent with the hypothesis that the NAD linked enzyme mainly converts glutamate to α-ketoglutarate and ammonia, while the NADP linked GDH is responsible for the reverse reaction (5, 15, 18). In cells grown on plain KC medium the energy requirements must be satisfied at least partly by oxidizing glutamate via α ketoglutarate and the tricarboxylic acid cycle, and the NAD linked GDH is stimulated. However, when the medium is supplemented with glucose, which is metabolized by means of the Embden-Meyerhof (10), the Entner Doudoroff, and the pentose phosphate pathways (11), before it enters the tricarboxylic acid cycle (8), abundant α ketoglutarate is supplied 'from above', from which glutamate may be synthesized by means of the NADP-linked GDH. Also the observation made by *Jyssum & Borchgrevink* (9) that NAD linked activity is stimulated in glutamate grown cells, indicates that this enzyme performs the oxidative deamination of glutamate.

In strain B 8152/66 glucose is not able to enter the metabolic pathways owing to a defect glucokinase (13). In this strain no effect of glucose is found on the GDH systems, as might be expected. It is somewhat unexpected that G 6 P does not have the same effect as glucose on the GDH, neither in strain B 8152/66 nor in strain M 6. However, in strain M 6 G 6 P is oxidized more slowly than glucose (10). It might be that when G 6-P is supplied from the medium it cannot enter the metabolic pathways as readily as glucose. The reason for this is not known.

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GLUTAMATE DEHYDROGENASES IN GENUS *NEISSERIA*

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The glutamate dehydrogenase system is examined in different *Neisseria* species The pH optima, temperature sensitivity, and the effect upon enzyme activity by adding glucose to the medium are compared to the findings in *N meningitidis* By agar gel electrophoresis it is shown that all species except *N catarrhalis*, *N ovis* and *N caviae* contain 2 different glutamate dehydrogenases, one linked to NAD and the other one to NADP The 3 species mentioned possibly contain only one glutamate dehydrogenase, reacting with both coenzymes The taxonomic implications of these findings are discussed

In *Neisseria meningitidis* two different glutamate dehydrogenases (GDH) are found, one coupled to NAD (NAD GDH) and one to NADP (NADP-GDH) (E C 1 4 1 2 and E C 1 4 1 4) (9) According to the author's knowledge nothing is known about GDH activity in other species of the genus *Neisseria* In the present report, the GDH system of several species within this genus is studied, and some of its properties are compared to those of *N meningitidis* Also the species *N catarrhalis*, *N ovis* and *N caviae*, which are proposed to be removed from the genus Neisseria (5, 8), are included, to see if these species also enzymatically differ from the "true neisserias"

MATERIALS AND METHODS

Neisseria strains The strains of the different *Neisseria* species are listed in Table 1 *N lactamica* 1379 and *N elongata* M 2 were isolated from the nasopharynx of presumably healthy recruits (3, 10) The strains were characterized culturally and biochemically according to *Berger* (1) and *Bøvre* (2)

Media Blood agar medium was used for preparing cell free extracts for the determination of GDH activity and for agar gel electrophoresis As this medium contained small amounts of glucose, it could not be used when the effect of glucose upon the GDH system was examined The KC medium, which was used in the experiments with *N meningitidis* (9), could be used only for *N catarrhalis*, as the other *Neisseria* species did not grow sufficiently on this medium Therefore, Mueller Hinton medium (Difco) supplemented with various concentrations of glucose was used in these experiments

Experimental procedures were as described previously (9) Additional procedures are described in the text

Chemicals DEAE-cellulose was purchased from Eastman Organic Chemicals, Rochester, New York, USA. Other chemicals were those used previously (9)

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RESULTS

GDH activity in different Neisseria *species* Table 2 shows the GDH activity in the strains of *Neisseria* included in the study All strains

TABLE 1 Neisseria *Strains Used in the Experiments*

Species	Strain	Source
N gonorrhoeae	1 a	AR
	2 c	AR
	44341	AR
	21308/70	KØ
	21319/70	KØ
	21332/70	KØ
N sicca	CN	UB
	6	UB
	8021	MJP/JB
N mucosa	M 1	UB
	M 4	UB
	M 9	UB
N perflava	ATCC 10555	AR
	A 2	UB
	191	UB
N flava	ATCC 14221	KB
	B	UB
	X 4	UB
N subflava	ATCC 11076	AR
	ATCC 19243	KB
	115	UB
N lactamica	ATCC 23970	REW
	1379	
	161 Sc	UB
N flavescens	ATCC 13115	KB
	ATCC 13117	KB
	ATCC 13120	KB
N cinerea	165/61	UB/KB
	137/62	UB/KB
	159/62	UB/KB
N elongata	M 2	
	7823/71	SDH
	8554/71	SDH
N catarrhalis	ATCC 8176	KB
	Ne 11	WBC/KB
	13016/62	KB
N ovis	199/55	KL/KB
	37/59	KL/KB
	917/60	KL/KB
N caviae	ATCC 14659	KB
	NCTC 10293	KB

The strains were received from AR Dr *A Reyn* KB Professor *K Bøvre* KL/KB from the collection of Dr *K Lindqvist* obtained through Professor *K Bøvre* KØ Dr *K Ødegaard* MJP/JB from the collection of Dr *M J Pelczar* Jr obtained through Dr *J Brundell* REW Dr *R E Weaver* SDH Professor *S D Henriksen* UB Professor *U Berger* UB/KB from the collection of Professor *U Berger* obtained through Professor *K Bøvre* WBC/KB from the collection of Dr *W B Catlin* obtained through Professor *K Bøvre*

possessed both NAD and NADP linked activity The NADP linked activities usually were higher than the NAD linked ones except in *N ovis* and *N caviae,* where they were almost similar In the non pathogenic *Neisseria* species the NAD linked activity tended to be higher, and the NADP linked one lower than the corresponding activities in *N meningitidis* and *N gonorrhoeae* There were, however, great differences between species and between different strains within the same species

pH optima pH optima were determined for GDH activities in extracts from different *Neisseria* species (Fig 1 Fig 2) In *N gonorrhoeae N sicca N subflava* and *N lactamica* the NAD linked activity had its optimum at pH 9 0–9 4, and the NADP linked one at pH 7 8–8 2 In *N catarrhalis* the pH optima for the NAD and NADP linked activities were 6 6 and 7 4 respectively In *N elongata* the two activities had almost similar pH optima, at pH 9 0 and 9 4 In *N flavescens* both activities had their optima at pH 8 2

Temperature sensitivity Extracts were placed in a water bath at the temperatures indicated for 3 minutes chilled in an ice water bath and examined for GDH activ

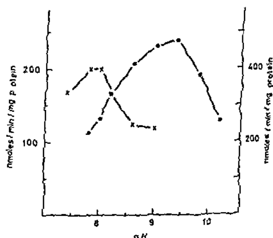


Fig 1 pH optima for GDH from *N gonorrhoeae* 1 a Left ordinate Oxidation of NADPH (x–x) Right ordinate Oxidation of NADH (●–●) Tris/HCl buffer was used at pH ≦ 9 0 glycin/NaOH buffer at pH ≧ 9 0

TABLE 2 *Glutamate Dehydrogenase Activity in* Neisseria

Species	Strain	Coenzyme			
		NAD	NADH	NADP	NADPH
N gonorrhoeae	1 a	7 2	3 5	55 7	583 4
	2 c	1 2	3 6	24 3	105 8
	44341	1 0	6 0	50 0	238 8
	21308/70	7 1	5 5	50 2	262 0
	21319/70	2 8	8 4	112 4	925 3
	21332/70	7 3	6 3	53 8	242 5
N sicca	CN	14 9	22 9	19 4	78 9
	6	17 4	28 2	21 7	227 2
	8021	14 7	73 5	25 7	
N mucosa	M 1	43 7	73 1	9 5	64 5
	M 4	3 6	11 2	20 6	87 7
	M 9	7 2	12 1	37 4	109 5
N perflava	ATCC 10555	28 5	69 7	40 5	194 1
	A 2	14.5	37 8	5 8	63 4
	191	25 5	31 6	20 4	102 0
N flava	ATCC 14221	23 1	86 6	16 3	126 3
	B	26 6	66 4	15 5	110 0
	X 4	12 8	54 0	5 2	76 1
N subflava	ATCC 11076	16 2	23 6	6 0	40 5
	ATCC 19243	11 5	17 7	13 8	48 7
	115	11 7	28 3	13 8	75 0
N lactamica	ATCC 23970	23 2	58 4	19 2	35 3
	1379	44 1	117 9	117 9	1082 3
	161 Sc	55 1	47 5	83 6	631 1
N flavescens	ATCC 13115	9 4	61 2	3 7	57 5
	ATCC 13117	12 2	1 3	27 5	72 0
	ATCC 13120	49 5	403 7	40 6	433 8
N cinerea	165/61	23 5	74 6	17 9	184 0
	137/62	147 1	382 7	124 3	672 4
	159/62	100 9	193 2	254 7	881 8
N elongata	M 2	6 9	4 4	50 2	118 1
	7823/71	6 8	0 4	11 3	68 8
	8554/71	2 9	2 4	49 1	101 0
N catarrhalis	ATCC 8176	0 8	46 4	15 5	278 7
	Ne 11	4 4	38 1	11 9	209 6
	13016/62	1 5	58 3	10 9	269 4
N ovis	199/55	5 6	32 4	15 4	43 8
	37/59	6 2	31 8	9 4	34 9
	917/60	2 5	36 5	9 4	47 2
N caviae	ATCC 14659	3 6	26 1	4 9	21 2
	NCTC 10293	5 9	30 7	3 9	20 9

Activities are expressed as nmoles substrate converted per min per mg protein

Fig 2 pH optima for GDH from different *Neisseria* species Enzyme activities are expressed as nmoles coenzyme oxidized (NADPH x – x NADH ● – ●) per minute by the volume of extract indicated for each species MOPS/NaOH buffer was used at pH ≦ 7 4 *Tris*/HCl buffer at pH 7 4 9 0, and glycin/NaOH buffer at *pH* ≧ 9 0 A *N sicca* CN NADH 50 µl, NADPH 25 µl B *N subflava* ATCC 19243 NADH 50 µl NADPH 50 µl C *N lactamica* 1379 NADH 100 µl NADPH 25 µl D *N elongata* M 2 NADH 500 µl, NADPH 25 µl E *N catarrhalis* Ne 11 NADH 150 µl NADPH 25 µl F *N flavescens* ATCC 13117 NADH 1000 µl, NADPH 25 µl

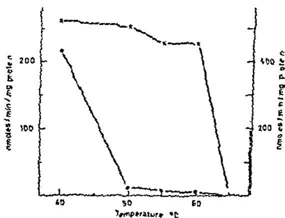


Fig 3 Temperature inactivation curves for GDH from *N gonorrhoeae* I a Left ordinate Oxidation of NADPH (x – x) Right ordinate Oxidation of NADH (● – ●)

ity (9) In *N gonorrhoeae* the NAD linked activity was rapidly destroyed at 50°C, while the NADP-linked activity was intact after 3 minutes at 60°C (Fig 3) In *N sicca*, *N subflava* and *N flavescens* the reverse appeared to be the case, the NAD linked activity being the most heat resistant one (Fig 4) though the difference in temperature sensitivity was far less pronounced than for the enzymes from *N gonorrhoeae* In the false neisserias' both NAD and NADP-linked activities seemed to be approximately equally affected

Effect of glucose on GDH activity In Fig 5 the influence of glucose concentration on the GDH activity in some species of *Neisseria* is shown In *N gonorrhoeae*, *N lactamica* and *N flavescens* the NAD linked activity

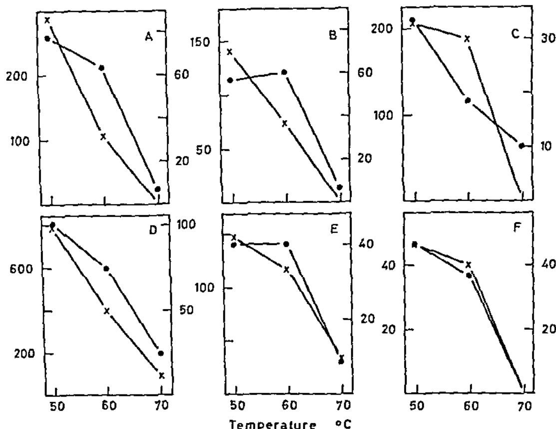


Fig 4 Temperature inactivation curves for GDH from different *Neisseria* species Left ordinates Oxidation of NADPH (x – x) Right ordinates Oxidation of NADH (● – ●) Enzyme activities are expressed as nmoles coenzyme oxidized per minute per mg extract protein A *N sicca* CN B *N subflava* ATCC 19243 C *N flavescens* ATCC 13117 D *N catarrhalis* Ne 11 E *N ovis* 199/55 F *N caviae* ATCC 14659

decreased as the glucose concentration was increased while the NADP linked activity increased In *N sicca* effect was found only on the NAD linked activity and in *N elongata* only on the NADP linked one, both of which changed in the same way as described for the corresponding activities in the 3 species mentioned above In *N catarrhalis* no effect of glucose upon the GDH activity was observed

Agar gel electrophoresis of GDH The migration of GDH relative to the NAD GDH of *N meningitidis* is shown diagrammatically in Fig 6 In *N gonorrhoeae* 2 c and *N elongata* 7823/71 no spot corresponding to the NAD linked activity could be seen although these strains have GDH activity coupled to both NAD and NADP (Table 2) Presumably the concentration of enzyme has been too low to be detected by this method In *N cinerea* 137/62 and in all 'false neisserias' the distance of migration was identical for both the NAD and the NADP linked activities In all other strains the two activities were clearly separated Except for *N gonorrhoeae* 2 c the distances of migration were similar in all strains of gonococci

In *N sicca*, *N mucosa*, *N perflava N flava*, *N subflava* and *N lactamica* the NADP linked activity mainly fell in 2 groups, one which migrated 10 per cent of the distance of *N meningitidis* NAD GDH, and another which migrated 40 per cent of that distance In *N mucosa* M 1 and *N lactamica*

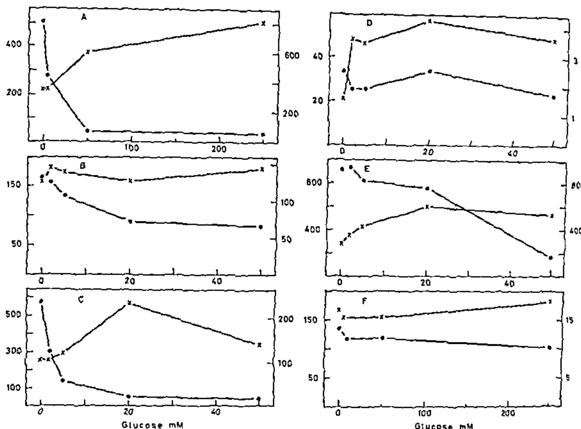


Fig 5 Influence of glucose concentration upon GDH activity in different *Neisseria* species Left ordinates Oxidation of NADPH (x – x) Right ordinates Oxidation of NADH (● – ●) Enzyme activities are expressed as nmoles coenzyme oxidized per min per mg extract protein A *N gonorrhoeae* 1 a B *N sicca* CN C *N lactamica* 1379 D *N elongata* M 2 E *N flavescens* ATCC 13117 F *N catarrhalis* Ne 11

161 Sc this enzyme behaved differently from that of the other saccharolytic *Neisseria* species (*N meningitidis, N gonorrhoeae,* and the 6 species mentioned above), as it migrated towards the cathode, like the corresponding enzyme from *N gonorrhoeae* 2 c

Greater variations were found regarding the NAD linked GDH In most of the saccharolytic *Neisseria* species other than *N meningitidis* and *N gonorrhoeae* there was variation in the distance of migration within one species, and there were also differences between the individual species

In *N flavescens* and *N cinerea* there were also intra- and interspecies differences in the electrophoretic pattern The 3 strains of *N elongata* seemed to comprise a homogenous group, as regards the NADP-GDH Also the NAD GDH had identical distances of migration in 2 strains, while it was not detected in the third one

The 3 species of "false neisserias seemed each to be homogenous as regards electrophoretic pattern Within each species the distances of migration were identical Both the NAD and the NADP linked activities seemed to cover the same distance of migration

Ion exchange chromatography of GDH from N catarrhalis Solid $(NH_4)_2SO_4$ was added to extract from *N catarrhalis* Ne 11 to bring it to 60 per cent saturation To the supernatant was added further $(NH_4)_2SO_4$ to 75 per cent saturation The precipitate was dissolved in 0 005 M Tris/HCl buffer pH 8 0 and dialysed for 24 hours against the same buffer The solution was applicated on a DEAE-cellulose coloumn (0 9 × 27 cm)

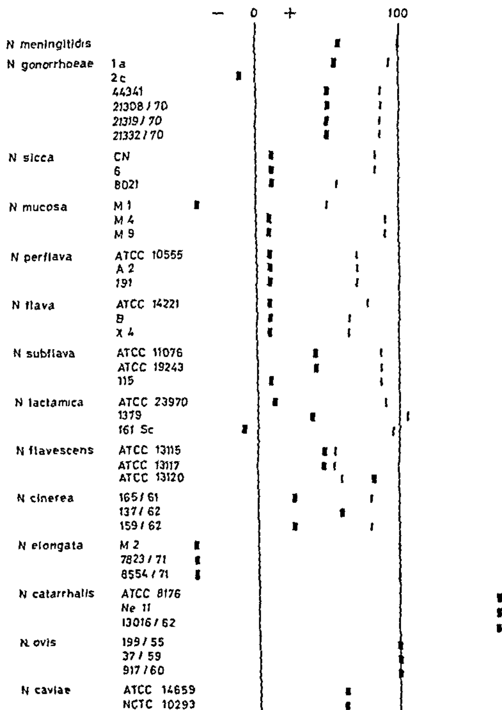


Fig 6 Electrophoretic pattern of GDH from different *Neisseria* species, diagrammatically O denotes the application point The migration distances are given relatively to that of the *N meningitidis* NAD-GDH, which arbitrarely has been given the value 100 The anode is to the right Heavy bars NADP-linked activity Light bars NAD linked activity In *N cinerea* 137/62, *N catarrhalis*, *N ovis* and *N caviae* the distances of migration of the NAD- and NADP-linked activities were identical

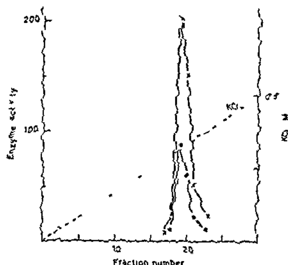


Fig. 7 Elution profile of chromatography of GDH from *N catarrhalis* Ne 11 on DEAE cellulose Left ordinate Oxidation of reduced coenzyme Activities are expressed as nmoles NADPH oxidized per minute per 0 25 ml eluate (x – x) and nmoles NADH oxidized per minute per 1 0 ml eluate (● – ●) Right ordinate concentration of KCl

which was equilibrated with the Tris/HCl buffer, and eluted with a linear gradient of KCl (0–0 5 M) in the same buffer Fractions of 3 ml were collected and examined for GDH activity (Fig 7) Peak activity was found in fraction 19 with both NAD and NADP as coenzyme The ratio NADPH/NADH roughly equalled 10 in all fractions 18 through 23, indicating that the 2 activities were eluted together

DISCUSSION

The GDHs from *N gonorrhoeae* seem to be quite similar to those found in *N meningitidis* Both have similar pH optima the reaction to heat is very much the same in the 2 species and in both the enzymes are affected in the same way by addition of glucose to the medium (9) Also by electrophoresis similarities are found although the *N gonorrhoeae* enzymes migrate slightly shorter than the corresponding enzymes in *N meningitidis* The distance of migration of the NADP GDH relative to that of the NAD GDH is however the same in the 2 species

The conformity in pH optima, thermal inactivation and electrophoretic pattern indicate that the glutamate dehydrogenases in *N meningitidis* and *N gonorrhoeae* are quite similar This again must reflect similarities in the base sequence of the DNA coding for GDH in these species, and is thus another indication of the close relationship between *N meningitidis* and *N gonorrhoeae*, as revealed by transformation of streptomycin resistance (5) and DNA hybridization (11)

There is a difference between the *N meningitidis*—*N gonorrhoeae* group on one hand and the remaining "true neisserias" on the other, as regards the heat stability of the GDHs In *N sicca*, *N subflava* and *N flavescens* the NADP GDH is destroyed at a lower temperature than the NAD GDH This probably reflects differences in protein structure It is possible that this difference does not involve the catalytic site as the pH optima are almost identical in *N sicca*, *N subflava*, *N lactamica*, *N meningitidis* and *N gonorrhoeae* The GDH in the "false neisserias" is probably different from those in the "true neisserias", as both activities in the former seem to be equally affected by heat and the *N catarrhalis* activities also have pH optima far different from those of the "true neisserias"

In *N lactamica* the effect of glucose on GDH is similar to that in *N meningitidis* and *N gonorrhoeae* In contrast to this is *N sicca*, where the NADP GDH is not affected The reason for this is not known In the non saccharolytic *N catarrhalis* none of the GDH activities are affected by glucose The GDHs in *N flavescens* and the NADP GDH in *N elongata*, however, are affected in the same way as in *N meningitidis* This is perhaps not surprising, as *Berger* (1) points out that *N flavescens* is not invariably indifferent to carbohydrates It may in some instances produce acid from several sugars and produces a polysaccharide from sucrose In addition it has been shown that *N flavescens* and *N elongata* contain some key enzymes in the degradation of glucose (*Holten*, manuscript in preparation) Their metabolism may

therefore be modified in the presence of glucose, and thereby induce changes in the activity of GDH.

Electrophoresis of enzymes has been used in attempts to clarify relationships between certain microorganisms. Electrophoretic patterns of lactate dehydrogenases (7) and glucose-6-phosphate dehydrogenase (12) in *Lactobacillus* have turned out to be relatively clear cut and have contributed to classification of these microbes. Greater variation was found in the electrophoretic patterns of glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase in enterobacteria (4). In 14 strains of *E. coli* 8 variants of the former enzyme and 11 of the latter were found, making the method more unreliable as a tool in classification in these bacteria.

Also in *Neisseria* there are variations in the electrophoretic patterns of the GDHs, but some information is obtained. *N. meningitidis* and *N. gonorrhoeae* are clearly different from the others. In *N. meningitidis* the patterns in the 12 strains examined were identical (9). In *N. gonorrhoeae* the number of strains examined is small, and hence does not permit strict conclusions, but it seems that the patterns are quite homogenous also in this species. How much the NADP-GDH in strain 2 c differs from that in the other strains is not possible to tell from the present results. However, the change in net charge from negative to positive suggests a relatively large change in the molecule without affecting its catalytic activity. The same applies to the NADP-GDH of *N. mucosa* M 1 and *N. lactamica* 161 Sc.

In *N. sicca*, *N. mucosa*, *N. perflava*, *N. flava*, *N. subflava* and *N. lactamica* there are some common features in the electrophoretic pattern. The NADP-GDH mainly falls in 2 groups (Fig. 6), except the enzymes from *N. mucosa* M 1 and *N. lactamica* 161 Sc which migrate towards the cathode. Seen in connection with the NAD-GDH this pattern is characteristic of this group of *Neisseria* species. In *N. flavescens*, *N. cinerea* and *N. elongata* the number of strains is too small to permit definite conclusions, but it seems that each of these species may form an entity of its own. The presence of 2 different GDHs indicate the relationship between these species and the other "true neisserias". *N. cinerea* 137/62 has an electrophoretic pattern identical to that of *N. caviae*, but studies on enzymes participating in the initial metabolism of glucose (*Holten*, manuscript in preparation) indicate that also this strain must be classified within the "true neisserias".

The patterns in *N. catarrhalis*, *N. ovis* and *N. caviae* indicate that the GDH in these species is fundamentally different from that of the other *Neisseria* species. Both the NADP- and the NAD-linked activities migrate exactly the same distance in electrophoresis. The 2 activities also react similarly to heat. Furthermore, the 2 activities in *N. catarrhalis* Ne 11 are eluted in the same fractions from a DEAE-cellulose column (Fig. 7). In *N. meningitidis* the 2 GDH activities are separated by chromatography on DEAE-cellulose (*Holten*, unpublished results). Although this is not conclusive evidence, it strongly suggests that *N. catarrhalis* Ne 11 contains only one GDH which may function with both NAD and NADP. This assumption is not contradicted by the fact that the NAD- and NADP-linked activities have different pH optima (13). One single GDH able to react with both NAD and NADP is previously known only to occur in *Mycoplasma laidlawii* among microorganisms (13).

This fundamental difference in the GDH system may further argument for the removal of *N. catarrhalis* from the genus *Neisseria* (5, 8), together with *N. ovis* and *N. caviae* (8).

The present results support earlier taxonomic work in genus *Neisseria* (summarized by *Henriksen* & *Bøvre* (8)). Also the GDH system indicates that *N. catarrhalis*, *N. ovis* and *N. caviae* should not be included in genus *Neisseria*. The non-saccharolytic species *N. flavescens*, *N. cinerea* and *N. elongata* make separate entities of their own within the genus. *N. sicca*, *N. mucosa*, *N. perflava*

N flava, *N subflava* and *N lactamica* comprise one group, which seems to be closer related to *N meningitidis* and *N gonorrhoeae* than to the non saccharolytic *Neisseria* species The pathogenic species *N meningitidis* and *N gonorrhoeae* are similar to each other, but clearly different from the other members of the genus, as regards the GDH system

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THREE NEW *ESCHERICHIA COLI* O ANTIGENS O154, O155 AND O156, AND ONE NEW K ANTIGEN, K94

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Three *Escherichia coli* strains representing strains from faeces and foods were established as antigenic test strains for three new *E coli* O groups, O154 O155 and O156 One of the strains, O154 was also designated as K antigen test strain for a new *E coli* K antigen K94

Among strains of *E coli* received at the International Escherichia Centre (WHO), Statens Seruminstitut, Copenhagen, a minority cannot be grouped with the available O and K test antisera When such untypable strains are considered of importance for diagnostic work because of a frequent occurrence and/or a possible causal relationship to human or animal disease from which they are isolated, they are established as test strains of new *E coli* antigens This paper reports about three such new test strains

MATERIAL

The three strains E1541 68 E1529 68 and F1585 68 were received at Statens Seruminstitut from Dr *B Rowe*, Central Public Health Laboratory London They were isolated during a study of travellers diarrhoea in British troops at Sharjah in the Trucial States Arabian Gulf E1541 68 accounted for almost 16 per cent of all *E coli* from food and was amongst the most common in the human faecal specimens E1529 68 and E1585 68 were common in both faeces and foods but not quite as numerous as E1541 68

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METHODS

Agglutination tests production of antisera, and methods for absorption were performed as described by *Kauffmann* (2) The precipitation test was a slightly modified form of the double diffusion in gel (7) using filter paper discs (10) Immunoelectrophoresis (IE) was carried out according to *Scheidegger* (8) The extracts used for both immune diffusion tests were prepared according to the principles described by *Orskov et al* (3)

Media Ox heart infusion broth was used as fluid and solid media (2) In addition the D5 medium (8) with 0 05 per cent glucose was used as solid medium (9)

RESULTS

Fermentation Reactions

The results of fermentation reactions and other biochemical tests are shown in Table 1

TABLE 1 *Biochemical Properties of Strains Examined*

	E1541 68	E1529 68	E1585 68
Adonitol	—	—	—
Dulcitol	+	—	+
Sorbitol	+	+	+
Arabinose	+	+	+
Xylose	+	+	+
Rhamnose	+[2]	+	+
Maltose	+	+	+
Salicin	—	—	+
Inositol	—	—	—
Lactose	+	+	+
Sucrose	+	—	+
Sorbose	—	—	—
Mannitol	+ +	+ +	+ +
Glucose	+ +	+ +	+ +
Indol	+	+	+
H S	—	—	—
Gelatin	—	—	—
Ammonium glucose	+	+	+
Ammonium citrate	—	—	—
KNO_3	+	+	+
Voges Proskauer	—	—	—
Methyl red	+	+	+
Urease	—	—	—
KCN	—	—	—
Malonate	—	—	—
Motility	+	+	+
Haemolysis	—	—	—
	O154	O155	O156

+ – positive after 1 day
+[2] – positive after 2 days
— – negative after 14 days
+ + acid and gas after 1 day

O Antigen Examination

For determination of O antigens overnight broth cultures heated at 100° C for 1 hour were tested for agglutination in the available O test antisera O1–O153 and O157, and in those prepared with the three strains under investigation Boiled broth cultures of all established O test antigen cultures were also examined in these three last mentioned O antisera

E1541 has reciprocal O antigen relationships to O1 and O64 but no depletion of the antisera takes place by cross absorptions Absorption of O antiserum E1541 with O64 removes antibodies against both O1 and O64 but the homologous titre is only lowered (640) In addition a minor one sided relationship to O129 exists as E1541 reacts in antiserum O129

E1529 does not react significantly in any O antiserum, while test antigens O19, O48 and O59 give titres of 640 in O antiserum E1529

With E1585 no cross reactions were detected with known O antigens

The three strains E1541 E1529 and E1585 are therefore established as test strains for new *E coli* antigens O154 O155 and O156, respectively

K Antigen examination

Examination for the presence of a K antigen was performed by IE and double diffusion in gel The IE results are shown in Fig 1 Extracts of bacterial suspensions heated to 60° C, 20 minutes and 100° C, 1 hour, were examined against homologous O and OK antisera In IE both 60° and 100° C extracts of strain E1541 showed against both O and OK antiserum an O line (or lines) adjacent to the application basin directed towards the cathode Furthermore a distinct arc was seen at a further distance from the basin but directed towards the anode with both extracts yet only against OK not against O antiserum

Corresponding to the picture in IE a heavy precipitation line was found in the gel diffusion experiment when the 60° and 100° C extracts were tested against OK antiserum and this line was missing in the O antiserum In addition the O and OK antisera showed 1 2 common O lines The antigen causing the line only against the OK antiserum is the K antigen

To test if this K antigen was already present among the known ones E1541 was examined by slide agglutination in the established *E coli* K antisera K1–K93 A positive reaction of significance was seen only in K90 Similarly the test strains for these K antigens were examined in OK antiserum of E1541 About 20 K test strains agglutinated to some

E1541-68 = 0154 K94,(K?) H4



E1529-68 = 0155 H9



E1585-68 = 0156 H47



Fig 1 Immunoelectropherograms of non heated and heated bacterial extracts against homologous O and OK antisera (schematic drawing) The troughs contain the homologous O and OK antisera The wells above the troughs contain unheated extracts i e 60° 20 extracts the wells below the troughs contain the heated extracts *i e the 60° 20 extracts heated to 100° C for 1 hour*

extent in this antiserum, but the only strong reaction was that of K7 E1541 also agglutinated in K7 antiserum but only weakly When 60° and 100° C extracts of E1541 were assayed in the gel diffusion test against antiserum K90 both extracts formed a weak line, but the line was not identical with any line formed by the test strain of K90

In antiserum K7 only the 60° and not the 100° C extract of E1541 precipitated E1541 68 has H antigen 4 like test strain K7, and the relationship to K7 was explained as an H antigen reaction (see also below)

When the OK antiserum of E1541–68 was absorbed by homologous boiled culture, some agglutinins were left the titres of which were dependent on the dilution state (undiluted or diluted 1 10) of the antiserum The same

TABLE 2 *O and OK Antiserum of 1541 Examined by Bacterial Agglutination Tests*

Antigen	O antiserum E1541	OK antiserum E1541		
		unabsorbed	absorbed by 100° C homologous culture	
			undiluted	diluted 1 10
E1541 live*	640	5120	512	5120
E1541 pril†	320	5120	16	5120
E1541 100° C§	5120	5120	—	<20
E1193 live	—	5120	<8	5120
E1193 pril	—	5120	—	5120
E1193 100° C	—	<20	—	—
Test strains of				
K7 live	—	5120	—	2560
K7 pril	—	<20	—	<20

* Living suspensions from broth agar plates formalinized

† Living suspension from broth agar plates supplemented with 0 1 per cent fluid pril, formalinized

§ Suspensions from broth agar plates heated to 100° C for 1 hour

TABLE 3 *Agglutination of Non Heated and Heated Culture in Homologous O and OK Antisera*

Antigen	O antiserum	OK antiserum	
		unabsorbed	absorbed by homologous culture heated to 100° C
E1529 live*	2560	≧5120	640
E1529 pril†	2560	≧5120	<20
E1529 100° C§	2560	≧5120	<20
E1585 live	160	≧5120	≧5120
E1585 pril	160	2560	<20
E1585 100° C	≧5120	2560	<20

*, † and § For explanation see Table 2

observation was reported earlier by *Ørskov & Ørskov* (6) From Table 2 it is seen that the antibodies left in the undiluted antiserum with all likelihood were H antibodies since E1541 failed to agglutinate or only agglutinated to a slight extent in this serum when the culture used for the agglutination test had grown on a plate supplied with Pril®, and it is known that pril inhibits H agglutination (1, 5) In contrast, the agglutination titre of *E1541* in the 1 10 diluted absorbed antiserum was not influenced by pril Similarly, another strain, E1193–68, also isolated during the same study, but not included in this report because it belonged to a known O group O129 gave a high titre in the 1 10 absorbed antiserum irrespective of whether the culture used was from an ordinary or a pril plate

The antigen causing the bilateral relationship between live culture of E1541 and the O129 strain (E1193–68) was not the H antigen, nor could it be related to the K antigen of E1541–68, K94 demonstrated above, as no cross reaction was found either in the IE or the gel diffusion examinations

It is further evident from the table that the reaction of the K7 culture in the 1 10 absorbed antiserum is highly influenced by addition of pril This finding fits with the fact that E1541 has the H4 antigen like test strain K7

According to the results OK antiserum of E1541 contains not only O154 and K94 antibodies, but also H4 antibodies and some directed against the unknown antigen common to E1541 and the O129 strain (E1193)

Examination in IE and double diffusion in gel of strain E1529 did not show the presence of a particular K antigen line By both methods the precipitation lines were identical in O and OK antisera and with 60° and 100° C extracts In IE the precipitation arc (or arcs) could be seen adjacent to the application basin on the anodic side In double diffusion in gel all tests showed one heavy line and 1–2 weak lines Unheated and heated *E1529* cultures were equally agglutinable in O antiserum i e no O inagglutinability was present The strain did not agglutinate in any of the established K antisera

By absorption of the OK antiserum of E1529 diluted 1 10 with homologous boiled culture no agglutinins were left for the homologous boiled or live culture after growth on a plate supplemented with pril (Table 3)

From these results it is concluded that E1529 has no special K antigen separable from the O antigen

Strain E1585–68 behaved similarly to E1529 in the respect that no difference could be demonstrated between the reactions of 60° and 100° C extracts in O and OK antisera by the IE or gel diffusion methods In the gel diffusion test two lines were formed against both antisera, and none of them could be characterized as heavy A distinct line was seen, however, in the OK antisera

from both immunized rabbits with the 60° C extract E1529 has H antigen 47 and the line just mentioned was identical with a line formed against antiserum for test H47 antigen From this fact and from agglutination experiments described below it was concluded that the line was an H antigen line

In OK antiserum of E1585 absorbed 1 10 with homologous boiled culture some agglutinins were left for live culture of E1585 and test strain for H47, when culture from pril plates was used (Table 3), no agglutination, however, was seen indicating that the agglutinins which remained were H agglutinins Live culture of E1585 agglutinates to a lower titre than boiled culture in the O antiserum expressing some degree of an O inagglutinability In spite of this it is concluded that E1585 has no K antigen as no such antigen can be demonstrated by any of the methods used

H Antigens

As already mentioned above, E1541-68 has H antigen 4, and E1585-68 H antigen 47 The H antigen of E1529-68 belongs to H9

To summarize we therefore propose the following serotype formulae for the strains examined

E1541-68	O154 K94, (K?) H4
E1529-68	O155 H9
E1585-68	O156 H47

DISCUSSION

In previous papers (3 4) on *E coli* O and K antigens the justification was discussed for a reconsideration of definitions of *E coli* capsule and envelope antigens and the reader is referred to those papers We proposed that numbering of new K antigens should be limited to antigens which serologically are easily differentiated from the lipopolysaccharide antigens Following this line only one of the three new O antigenic test strains dealt with in the present paper has such an antigen However this strain has not only one but two antigens separable from the O antigen but only one of them, K94, was numbered This antigen is easily demonstrable by one of the precipitation methods also after heating to 100° C, therefore it is reasonable to think that it is a polysaccharide K antigen The other antigen distinguishable from the O antigen, but less easily, was only demonstrated by the bacterial agglutination technique As no further examination of this antigen has been carried out, we prefer for the time being to point to its presence but to keep it unnumbered Several such antigens exist, most of them are probably of protein nature *e g* fimbrial antigens, and some are common antigens which are unnumbered as yet This type of antigen may easily disturb the common serotype determination as carried out by the agglutination technique This difficulty can only be overcome by introduction of additional techniques such as precipitation by double diffusion in gel or immunoelectrophoresis At this juncture we would stress the influence which H antigens may have on examinations for surface antigens, a fact which was true with all three strains in the present paper That *E coli* OK antisera often contain H antibodies was reported by *Wright* & *Villanueva* (11), but the frequency with which the H antigen is developed on solid media is probably not realized by all investigators The inconvenience of H agglutination in diagnostic and research work can be overcome by incorporation of small amounts of pril or similar compounds

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TYPING OF *HERPESVIRUS HOMINIS* STRAINS BY INDIRECT IMMUNOFLUORESCENCE AND BIOLOGICAL MARKERS

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An indirect immunofluorescence technique was applied to the typing of *Herpesvirus Hominis* (HVH) strains of genital and non genital origin The virus type was determined from aceton fixed cells with rabbit convalescent sera and anti rabbit conjugate Results from kinetic neutralization confirmed the results Cytopathic effect and plaque characteristics in Vero and chick embryo cell monolayers were evaluated for their usefullness in distinguishing type 1 strains from type 2 strains Cytopathic effect was found to be a less reliable marker even under standardized conditions. On the other hand the plaque size in Vero cells and the plaque forming capacity in chick embryo cell monolayers discriminated the two types

Cross reaction between the two antigenic types of *Herpesvirus Hominis* (HVH) is extensive and various modifications of the neutralization test are needed for the serological typing (19) Immunofluorescence permits a rapid and simple identification of virus isolates and a direct modification has been applied to the typing of HVH strains (8 To overcome the unfavourable cross reaction *Geder and Skinner* (3) have developed a technique which differentiates the two types by membrane fluorescence with sorbed sera The greater sensitivity of indirect immunofluorescence compared to direct methods enables the differentiation of typespecific herpesvirus antibodies in human and rabbit sera (5) and this simple method is also applicable to the typing of HVH isolates Intratypic HVH strains are however antigenically heterogenic (20), and the combination of serological typing with biological markers would offer a reliable method for herpesvirus typing in diagnostic work In this report the behaviour of genital and non genital strains was studied with respect to the cytopathic effect and plaque characteristics in Vero and chick embryo cell monolayers and a combination of immunofluorescent typing and these markers was successfully used for HVH typing

MATERIAL AND METHODS

Viruses 19 strains isolated from patients with genital herpetic infections herpetic eczema stomatitis and kerato-conjunctivitis and reference strains Tyler 115 F (type 1) and Curtis E 304 (type 2) (9) were used in this study All other strains except one (No 7) had been isolated in continuous monkey kidney cells without passage cultivation and stored at –70° C until used Strain

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No 7 had been isolated in human embryonic skin cells and stored in the same way The strains were inoculated into BSC 1 cell monolayers and when distinct cytopathic effect (CPE) was observed (usually overnight), the cells and supernatant were collected for immunofluorescent typing and for further characterization of the strain with biological markers

Plaque assays After one cycle of freezing and thawing the virus was clarified with low speed centrifugation and assayed in cultures of Vero and primary chick embryo fibroblast (CEF) cells in 50 mm plastic dishes 0 2 ml of serial tenfold dilutions were inoculated to washed monolayers and after 1 hour s absorption the inoculum was removed 5 ml carboxymethylcellulose overlay (18) was added and the cultures were kept at 37° C in CO atmosphere for three days Then the overlay was removed and replaced with 3 ml crystal violet stain for 15 minutes and rinsed with water

Immunofluorescent typing Infected cells were washed twice with PBS and dispersed on two slides four ringed spots on each After air drying cells were fixed in acetone for 3 min at +4° C The slides were dried again and washed 10 min in phosphate buffered saline with continuous stirring and then dipped in distilled water The first spot was stained with anti type 1 rabbit anti herpes serum in a dilution that gave positive reaction only with type 1 antigen The second spot was stained with anti type 2 rabbit serum in a dilution that gave positive reaction with both type 1 and type 2 antigens The third spot was stained with the conjugate only and the fourth spot was left unstained The conjugate used was commercially available anti rabbit gamma globulin of swine origin (Sevac, Prague) with amido black as a counterstain (5)

To determine the working dilutions of the rabbit anti herpes sera immunofluorescent antibody titres were determined for both homologous and heterologous herpesvirus types (5) Type 1 anti serum was used close to its endpoint (mostly 1/500) and type 2 antiserum 2 to 4 times stronger than its heterologous titre (mostly 1/50)

Kinetic neutralization The following modification of the technique was used (15) 2000 plaque forming units of virus were incubated with rabbit convalescent antiserum in pre established dilution in a 37° C water bath Samples were taken immediately after mixing the virus and the serum and after 5 min and 30 min incubation The neutralization was stopped by diluting the sample 50 fold in ice-cold PBS and the surviving virus was assayed in Vero cell monolayers in plastic dishes In each neutralization experiment the reference virus strains were included

The antisera used in kinetic neutralization and immunofluorescent typing were derived from rabbits infected by corneal scraping as described earlier (5)

RESULTS

Cytopathic effect The CPE produced by the strains was registered when distinct changes were observed in BSC 1 cell monolayers It was classified in two different forms of degeneration The first was characterized by small rounded cells in clusters and called arbitrarily "type 1" CPE In the other form syncytia and large cells dominated and this was called "type 2" CPE As the results in Table 1 show, this marker permitted only preliminary orientation, in four cases CPE was misleading

Plaque production in Vero and CEF cells In Table 1 the proportion of plaque counts in Vero and CEF cells is presented Most type 1 strains were inefficient plaque producers in chick cells while type 2 strains produced equal or even higher amounts of plaques in chick cells compared to Vero cells Nevertheless, some of the type 1 strains produced plaques with rather high efficiency in CEF (Nos 1, 5 and 9) showing the biological divergence of the strains The most constant marker was the plaque size in Vero cells all type 2 strains produced large plaques, while type 1 strains produced small plaques

Immunofluorescent typing 11 of the strains gave in aceton fixed BSC 1 cells a type 1 staining pattern reacting with both sera, while 8 strains were positive with type 2 antiserum only In some cases type 2 infected cells reacted weakly with anti 1 serum but this was always clearly distinguishable from the bright fluorescence produced in type 1 infected cells with this serum When other cells than BSC 1 were tried for typing more variation in the staining pattern was observed This points to the importance of the use of the same cell type for typing where the reagents have been standardized

Neutralization kinetics confirmed the results of immunofluorescent typing Type 2 antiserum prepared against the reference

TABLE 1 *Cytopathic Effect, Plaque Forming Capacity in Chick Embryo Cells (CEF), Plaque Size in Vero Cells and the Results of Immunofluorescent (FA) Typing of Herpesvirus Strains Type 1 and Type 2*

	Strain No	Type of CPE		PFU Vero/CEF	Plaque size in Vero	FA typing	
		1	2			anti 1 serum	anti 2 serum
type 1 strains	1	+	—	6	small	+	+
	2	+	—	1900	small	+	+
	3	+	—	>2500	small	+	+
	4	+	—	>2500	small	+	+
	5	+	—	2	small	+	+
	9	—	+	10	small	+	+
	10	+	—	1000	small	+	+
	13	—	+	>8000	small	+	+
	15	+	—	200	small	+	+
	16	+	—	2700	small	+	+
	19	+	—	1200	small	+	+
	21*	+	—	1100	small	+	+
type 2 strains	6	—	+	1.0	large	—	+
	7	—	+	1.0	large	—	+
	8	—	+	1.0	large	—	+
	11	—	+	1.0	large	—	+
	12	+	—	0.4	large	—	+
	14	—	+	1.4	large	—	+
	17	+	—	0.9	large	—	+
	18	—	+	0.3	large	—	+
	20*	—	+	0.5	large	—	+

* reference strains

strain neutralized 8 of the strains better than type 1 antiserum while 11 of the strains neutralized effectively with type 1 antiserum only. The K values with type 1 antiserum varied between 1.3 and 2.2 for type 1 strains and 0.6 and 1.1 for type 2 strains and with type 2 antiserum between 0.8 and 1.2 for either types.

DISCUSSION

The epidemiology of HVH type 2 has received interest since *Nahmias et al* (11) and *Rawls et al* (14) observed the association between cervical malignancy and herpesvirus type 2 antibodies. Neonatal herpetic infection is caused by this type (10, 16) and the finding that this disease perhaps is more common than previously believed (12) has also increased the need for the virological diagnosis of HVH type 2 infections. The isolation of herpes simplex virus is easy, but the strong antigenic cross reaction between the two types makes the serological typing inconvenient. Immunofluorescence has been used for the typing of HVH strains. *Nahmias et al* (8) used infected HEp 2 cells and found the same one way cross reaction in endpoint titrations that is evident from neutralization techniques in his immune sera. Another group has reported highly specific results by using unfixed cells as antigen (3). Sera were absorbed with cells infected with heterologous HVH type and after absorption they reacted only with homologous antigen on the membrane of unfixed cells.

Indirect immunofluorescence differentiates typespecific herpesvirus antibodies in human and rabbit convalescent sera (5), and by using appropriate dilutions the typing of isolated strains can be conveniently and rapidly done. The typing can be completed

in less than two hours, and no absorptions of the immune sera are needed The non specific staining can be eliminated by the high dilutions of immune sera and by the use of a counterstain in appropriately diluted conjugate In this work a complete accordance between immunofluorescent typing and kinetic neutralization was observed However, the heterogeneity of different herpesvirus strains within the types (20) makes the use of biological markers desirable Some authors consider the type of CPE as a characteristic marker (4, 7, 13), while others emphasize the effect of passage history, host cell, etc to the CPE observed (1) In this study the cytopathic effect only partially supported subsequent typing results in spite of similar passage history and the same host cell

The plaque morphology in Vero cells was observed to be a constant characteristic in differentiating the types However, *Smith et al* (17) found that both type 1 and type 2 laboratory strains produced small and large plaques, and from both, small and large plaque variants could be cloned Their cloned strains retained the plaque morphology in rabbit cornea cells even after serial passages These experiments showed that their strains, although antigenically homogeneous, consisted of clones with different plaque characteristics In our freshly isolated strains variation in plaque morphology was observed only to some extent type 2 strains showed some heterogeneity in plaque size However, the dominant plaque form with all type 2 strains was 2-3 times larger than the predominant plaque produced by type 1 strains By comparing the plaque morphology with the reference strains in each experiment, it was easy to distinguish type 1 strains from type 2 strains The plaque forming efficiency in chick embryo cells divided the strains into two groups This confirms the observations of others (2, 6) The inability to synthesize DNA has been reported to be the reason for the failure of type 1 strains to produce plaques in chick cells However, in their freshly isolated oral strains *Lowry et al* (6) found a small fraction of virus capable to form plaques This fraction varied from 10^{-2} to 10^{-6} In our study three type 1 strains were more effective plaque producers in CEF monolayers than their strains They produced only 2 to 10 times more plaques in Vero than in CEF cells One explanation for this discrepancy might be the effect of the original host cell, two of these strains were isolated from conjunctival samples, one from a vesicle of a patient with herpetic eczema Nevertheless, all other strains tested could easily be divided into two types, and even these "atypical" type 1 strains were different from the type 2 strains

The combination of immunofluorescent and biological typing permits a reliable typing for even atypical HVH strains It is simple and can easily be applied to routine diagnostic work

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EPIDEMIOLOGICAL MARKERS FOR *PSEUDOMONAS AERUGINOSA*

I Serogrouping, Pyocine Typing—and their Interrelations

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Experience with serogrouping and pyocine typing of *Pseudomonas aeruginosa* has been presented The Habs serogrouping system was compared with the schemas of *Homma et al*, of *Lanyi*, and of *Sandvik* All the reference strains of *Homma et al* were groupable by the Habs set, likewise most of the Lányi strains The bovine *Sandvik* serogroup reference strains all had a Habs counterpart, except O II which was incorporated in the Habs system as O 13 The bovine serogroup O VIII corresponded to Habs O 8 It was considered justified to recognize the Wahba O 14 as a separate entity The pyocine typing method of *Gillies & Govan* was suitable since it rendered a reasonably reproducible result With this procedure, 96 7 per cent of the strains were typed The major disadvantage of the method was its insufficient subdivision of the *P aeruginosa* strains Only three types constituted 65 3 per cent of the material The data suggested a correlation between certain serogroups and pyocine types

The importance of having a dependable typing procedure for *Pseudomonas aeruginosa* is emphasized by the increasing frequency of nosocomial pseudomonas infections As typing procedures, one may employ serogrouping bacteriophage typing, or pyocine typing

Serological characterization has been attempted before the two other techniques were introduced The first serogrouping system to gain recognition appears to be the system of *Habs* (10) The 12 Habs O groups have subsequently been supplemented by the serogroups O 13 identified by *Sandvik* (24, 29) (as O II) and the O 14 of *Wahba* (29) Other candidates for inclusion in the serogrouping have appeared in the literature but at the time when serogrouping was introduced in this laboratory in 1965, the above modifications represented the most advanced alternative available

Below, this schema of 14 O groups will be compared with the pyocine typing procedure of *Govan* (8) and *Gillies & Govan* (7)

In this context, a few technical details of the pyocine typing procedure and serogrouping method in addition to the interdependence between the Habs schema and three other serogrouping systems shall be elaborated

MATERIALS AND METHODS

Serogrouping

The Habs type strains O 1 O 12 (10 were kindly made available by *Dr G Quincke* Hygiene-Institut der Universitat Heidelberg Heidelberg W Germany As O 13 was obtained the group II strain (24) from Professor *O Sandvik* the Veterinary College of Norway Oslo Group O 14 type

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TABLE I *Cross Reactions between Grouping Sera for* Pseudomonas aeruginosa *in the System Built on the Habs Schema (10) Titres Obtained with Unabsorbed Sera by the Tube Agglutination Method Using Boiled Antigen*

Serum	Antigen 1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	640													
2		320			10									
3	10		640											
4				400										
5		10			320									
6	20					320			160	10				20
7				10	10	10	320	20						
8							400	400						
9	160					20			640	160				20
10	80								160	640				80
11	10					20	10	10	20	20	640	10	10	20
12												800		
13												10	640	
14		50				200							200	160

strain was the Wahba O 14 type (29) which was obtained through the courtesy of Dr *M T Parker*, Cross-Infection Reference Laboratory, Colindale, London, U K The reference strains of Lányi's O groups (16) were generously furnished by Dr *B Lányi*, National Institute of Public Health, Budapest, Hungary, The *Homma et al* (12) type strains were procured through the assistance of Dr *M Kudo*, the Institute for Infectious Diseases, the University of Tokyo, Tokyo, Japan

The sera were produced by immunizing rabbits with suspensions of bacteria (grown on Tryptose Blood Agar (Oxoid) for 18 hours at 37° C) which had been heated in boiling water for 2½ hours, washed 3 times in sterile saline and the density adjusted such that a 1/10 dilution rendered an optical density of 0 10-0 12 on a Beckman DB spectrophotometer with a medium slit at 760 nm The animals were injected every 3 days with increasing amounts of antigen intravenously according to the schedule 0 1 0 2 – 0 4 0 8 – 1 6 – 2 0 2 0 ml Between injections the preparations were kept at 4° C A high titered serum from one rabbit was used for each sero-group during serogrouping

Absorption of cross reacting antibodies was done with an antigen prepared by heating a suspension of an over night blood agar culture in 0 85 per cent saline for 2½ hours in boiling water with subsequent washing 3 times in sterile saline The pellet from the last centrifugation was mixed thoroughly with an equal volume of rabbit serum Incubation was first for 5 hours at room temperature with periodic resuspension and then over night at 4° C Completeness of absorption was controlled by a tube technique where 0 5 ml of an antigen suspension was mixed with 0 5 ml of the serum dilution and incubated at 37° C (water bath) over-night

The cross reactions observed with crude sera are apparent from Table 1 Factor sera were produced by mutual absorption between the following group pairs 2 and 5, 6 and 14, 7 and 8, 9 and 10 For typing purposes were used dilutions which were 3–10 times more concentrated than the homologous titres

Agglutination was performed on chemically clean disposable slides employing live suspensions of over night blood agar cultures The antigens were prepared immediately before use as dense saline suspensions

As the initial step in grouping, the cells were tested against three serum pools consisting of the following partly cross-reacting crude group sera

Pool I 1, 3, 9, 10, 11
Pool II 6, 7, 8 13, 14
Pool III 2, 4, 5, 12

After reaction of a strain with a pooled serum it was tested with each of the factor sera within the pool to identify its specific group

Pyocine Typing

The procedure of *Gillies & Govan* (7) was followed with minor modifications The medium used was Tryptone Soya Agar (Oxoid) with supplements of 0 08 per cent $CaCl_2$ and 5 per cent whole, defibrinated horse blood Pyocine production took place at 32° C for 14 hours The indicator strains, generously supplied by Dr *J R W Govan*, Department of Bacteriology, University of Edinburgh Medical School, Edinburgh, U K., were used as

1 100 suspensions of 3–4 hour broth cultures after incubation in a gyratory shaker at 37° C Growth of the indicators on the typing plates proceeded for 14 to 18 hours before reading The indicators were inoculated with a set of loops fixed at appropriate distances in a bar holder (corresponding to a contrivance used by Govan (Personal communication, 1967))

Strains of P aeruginosa

486 of the strains were the same as described in detail in a previous communication (2) For assessment of the pyocine type reproducibility, the 23 O group reference strains of Lányi were also included

RESULTS

I. Serogrouping

1 1 Cross Reactions Observed in Different Laboratories

The serological reference strains and the sera produced against them in different labo-

TABLE 2 *Reactivity of* Pseudomonas aeruginosa *Group Sera and Antigens Found by Different Investigators*

	Habs (10)	Sandvik (23, 24, 25)	Wahba (29)	Mikkelsen (20)†	Mikkelsen (21)£	Bergan**
Type serum*						
1		11	3		6	
2	5	–	5		5	5
3			1,4	1,11		1
4		3,13§	3 6,9,11	11	11	
5	2	–	2	2	2	2
6			2 4,5,13,14	1	1	1,9,10 14
7	8		8	All antigens	8	4,5,6,8
8		–	7	7	7	7
9		11§	4			1,6,10,14
10						1,9,14
11		9	4		4	1 6,7,8,9 10,12,13,14
12		–				
13	–$		3,6,10,14	–		12
14	–	–	6	–	–	2 6,13
Type strain						
1			3	3,6 7		3,6,9,10,11
2	5	–	5,6	5 7		5 14
3		4§	1,4,13	7		
4			3,6,9,11	7		7
5	2	–	2,6	7		2 7
6			4,13,14	7		7 9,11,14
7		–	8	8		8 11
8	7		7	7		7 11
9		11	4	7		6 10 11
10		–	13	7		6 9 11
11		9§ 1	4	3,4,7		
12				7		11 13
13		4§	6	–		11 14
14	–		6,13	–	–	6 9 10,11

† Immunization of Experiment 8, immunization technique b except serum 6 and 9 where immunization technique c has been employed
£ Cross reaction table ot given but indication of cross reaction between 1–6, 2–5, 4–11 and 7–8
** The results of this investigation
* The nomenclature of the Habs strains have been used for reference
§ The reactions were weak
$ The corresponding str n or group serum was not available

TABLE 3 *Correspondence Between the Serogroups of the Habs (10) Schema and of other Schemas (12, 16, 24, 25)*

Habs	Homma et al	Lányi*		Sandvik
1	B	U72/59	(6)	VII
2	A, C	PS340	(3c)	
3		Ps304	(1)	III
4		Ps898	(11)	IV
5	A, C	Ps11	(3ab)	
		Ps317	(3ad)	
6	D	Ps469	(3adc)	I
		Ps323	(4ad)	
		Ps161	(4ab)	
7		U645	(5abc)	
8		U118/59	(5abd)	VIII
		Ps194	(5ad)	
9		Ps196	(10a)	V
		Ps275	(10ab)	
10		Ps21	(2)	
11		U118/59	(7ab)	VI
		Ps415	(7ac)	
12		V142a	(13)	
13				II
14				

* The numbers in parentheses indicate serogroup specificity according to Lányi's schema

Agglutination of live antigens by slide technique

The Lányi reference strain Ps217 (4ac) was self agglutinable, the strains Ps48 (3df), U900/60 (8), Ps910 (9), and L83 (12) were not grouped by the present sera The correlations of the Habs groups and the Sandvik groups I and III–VII were as shown by *Sandvik* (24)

ratories have not been entirely in conformity with each other (Table 2) There are some striking discrepancies from one set to another

For instance, with the O 4 group serum *Habs* (10) found no cross reaction, *Sandvik* (23, 24) observed weak reactions with O 3 and O 13 at a dilution of 1/10, *Wahba* (29) found agglutination of the groups 3,6 9, and 11,*Mikkelsen* (20) noted agglutination with O 1 in one instance and with O 11 in another (21) With serum O 11, *Habs* (10) and *Mikkelsen* (20) noted no cross-reactivity, whereas *Sandvik* (23, 24) agglutinated O 9 cells, *Wahba* (29) and *Mikkelsen* (21) the O 4 antigen and the author observed a whole series of reactions, *viz* with the groups 1, 6 7, 8, 9, 10, 12, 13, and 14

With the O 4 antigen, neither *Habs* (10), *Sandvik* (23, 24), nor *Mikkelsen* (21) had any reaction The antigen was agglutinated by four of Wahba's sera and in my and *Mikkelsen*'s (20) system by group serum O 7

1 2 Relationship Between Reference Strains from Other Typing Schemas as Evidenced by Grouping with the Habs Group Sera

In order to compare the typing schemas with which the author has had occasion to get acquainted, the available group strains of

TABLE 4 *Cross Agglutination with Sera Prepared, against Habs (10) Serogroups O 1,2,5, and 6 and Homma et al (12) Serogroups*

		Antigens							
		Habs				Homma et al			
		1	2	5	6	A	B	C	D
Sera									
Habs	1	640					320		
	2	20	160	20		160		40	
	5		10	320		160		40	
	6	20			320		20		320
Homma et al	A		20	320		320		160	
	B	640					640		
	C		160	40		160	10	160	
	D	40	10		320		320		320

Tube agglutination with 2½ hours boiled antigens.

other schemas were tested with the Habs sera (Table 3) Sera were made with the Homma et al (12) strains, but not with the Lanyi (16) or the Sandvik (23, 24) strains The cross reactions with Homma and Habs sera and antigens are shown in Table 4 In the Habs system, the O 2 and O 5 cross-react This is reflected in the behaviour of the *A* and *C* group strains The Homma O-group *A* perhaps corresponds to O 5 and the O-group *C* to O 2 as evaluated from the titres of the Habs O 2 and O 5 antigens in Homma *A* and *C* sera, but both Homma group strains were agglutinated to the same titre in Habs group sera and no absorption has been carried out to clarify the point The identification of Homma O group *B* as a Habs O 1 and O group *D* as a Habs O 6 appears unequivocal

The O VIII type strain defined for Sandvik's schema by *Thörne* & *Kyrkjebo* (25) completely absorbed serum made against Habs O 8

1 3 Relationship Between O 6 and O 14

Table 1 shows that the serogroups O 6 and O 14 cross-react The reactions reported in Table 1 were obtained with cells treated for 2½ hours at 100° C The titre of O 6 serum against live O 14 antigen is higher As the grouping was presently done with live antigen, the latter result is more pertinent to the results of this communication in terms of the serological cross reactivity between the two groups Clearly O 6 and O 14 were closely related Whether to consider them too much alike to give them different group designations, depends upon the result of cross absorptions In Table 5 is indicated that homologous antibodies remain after heterologous absorption These distinctions between strains were considered sufficient for a recognition of both O 6 and O 14 as separate serogroups

TABLE 5 *Cross Absorption Results of O 6 and O 14*

		Agglutination titre with antigen	
		O 6	O 14
Serum O 6	Un absorbed	320	320
	Absorbed with O 14	40	0
Serum O 14	Un absorbed	320	540
	Absorbed with O 6	0	320

Tube agglutination with *live antigen*

1 4 Frequency of Individual Serogroups

Table 6 shows the distribution of the pseudomonas* strains between the different O groups It is apparent that the groups 3, 5, and 6 are the major entities with 18 1 10 3, and 17 5 per cent of the strains each Thus, these three groups alone make up almost half of the strains In a few instances in spite of using factor sera which only reacted with the homologous type strain, cells reacted with two factor sera The cross reacting entities O 6 and O 14 comprise 27 0 per cent and the entities O 2 and O 5 contain 17 1 per cent A portion of the strains were self-agglutinable, 7 4 per cent, and 6 2 per cent did not react with any group serum

2 Pyocine Typing

2 1 Attempts to Improve the Test Technique

2 1 a Limitations of previous pyocine typing systems The experiences with another pyocine typing method which employed a different set of indicator strains and different cultural conditions had been so discouraging (1, 8), that it was considered necessary first to establish the limitations of the technique of *Govan* (8) and *Gillies* & *Govan* (7)

2 1 b Govan pyocine typing procedure

2 1 b α Dilution of indicator strain cultures As has been demonstrated previously, the density of the suspension of indicator strains is important for the successful typing (1) Parallels with undiluted 3 hour vigorously aerated broth cultures and dilutions of 1/50 1/100, and 1/150 clearly showed that the

* This series of articles uses the terms pseudomonas and *P aeruginosa* interchangeably

TABLE 6 *Correspondance of Serogroups and Pyocine Types for 486 Strains of* Pseudomonas aeruginosa

	Pyocine types§															
	None	1	3	5	8	9	10	11	17	22	27	29	38£	Misc †	Sum	Sum %
	3	11	4	2			1			1		1		7	30	6 2
	5	13	4	3			1	1	1	1				7	36	7 4
	3	2	3			1	2	4						12	27	5 6
	1	22	3				3							1	30	6 2
		8	8	48	1	5	1	1		1		5	2	8	88	18 1
		4	0	1			1							1	7	1 4
		34	5	1			6	1	1					2	50	10 3
	1	6	56	4				2		2	3	1	1	9	85	17 5
		2					2								4	0 8
		2	1				9								12	2 5
	1	22	3			1	1					1		1	30	6 2
		6	2	4			1			1					14	2 9
		12					3							1	16	3 3
		2													2	0 4
		1	3									1			5	1 0
	1	2	2		6			1	2		1			12	27	5 6
	1	2	3	3	1				2		1		1	5	19	3 9
*		3											1		4	0 8
	16	154	97	66	8	7	31	10	6	6	5	9	5	66	486	
%	3 3	31 7	20 0	13 6	1 6	1 4	6 4	2 1	1 2	1 2	1 0	1 9	1 0	13 6		

§ The following pyocine types of Gillies & Govan were missing in our strains 2 4 7 12 13 18 20 21 23 24 25,26 28 32 34,36,37

* The Miscellaneous serogroups were (i) one culture reacting with the four factors O 7 8 9 and 10 belonging to pyocine type 38 and (ii) three strains reacting with both O 2 and O 5 factor sera belonging to pyocine type 1

† The Miscellaneous pyocine types contained 1–4 strains each

$ SA = Self agglutinable £ The pyocine type 38 had the inhibition pattern — + + — — — + —
Cells agglutinated as live antigens by slide technique

technique was optimally sensitive with dilutions of 1/100 or 1/150

2 1 b β Primary incubation temperature for pyocine typing

Govan (8) clearly showed that pyocine production was better at 32° C that at 35 5 or 37° C Since pseudomonas grows both below and above that range 5 strains of different pyocine types were typed after primary growth and pyocine production at 30 32 33 35 and 40° C (2 parallel plates at each) Two strains showed identical patterns in all instances These belonged to the frequently occurring pyocine types 1 and 5 With the others the same reactions appeared after growth at 30 32 and 33° C

2 1 b γ Supplements of calcium and magnesium

These bivalent ions being of importance for the absorption of several pseudomonas bacteriophages it was considered that an improvement in pyocine typing reactions might also result after adding Ca and Mg A series of experiments on 87 different pseudomonas strains with Ca and Mg (0 08 per cent) singly and in combination was set up to elucidate the question

The nature of the pyocine inhibition zones makes it difficult to quantitate the results Still there are indications that calcium improved reactions since more indicators were inhibited and the inhibition reactions were easier to read with calcium In many instances, though there was no discernible difference between the control and the plate with Ca Magnesium appeared never to im

TABLE 7 *Reproducibility of Pyocine Typing Assessed by Two Typings of 509 Strains*

Identical patterns	Difference in the number of indicators listed				Total
	1	2	4	5	
488	14	4	1	2	509
	21 strains				

prove results Mg had a negative effect in a few instances when alone, but not when in combination with Ca A combination with Ca and Mg *was no improvement* as the plate with only Ca was better to interpret in some instances

2 1 b 8 Time of primary incubation

The period of primary incubation is critical Only 8 hours was insufficient 14–18 *hours* optimal whereas 24 hours for some strains was associated with loss of indicator inhibition

2 2 *Reproducibility of Pyocine Typing* ad modum Gillies & Govan *(7)*

With the modifications documented above (cfr Materials and Methods) the reproducibility of the procedure was tested on 509 strains of *Pseudomonas aeruginosa* by typing each strain twice on the same day As seen in Table 7 only 21 strains (4 per cent) showed aberrant patterns

Moderate secondary growth within the inhibition zone was found in some positive reactions but diffusely inhibited growth was recorded as a negative reaction The interpretation of *reactions required* some experience

2 3 *Frequency of Pyocine Types*

Table 6 shows the distribution of pyocine types amongst 486 strains It is apparent that the types 1 3 and 5 cover approximately 2/3 of all strains Only 3 3 per cent were non typable by this procedure

3 Correlation between Serogrouping and Pyocine Typing

The comparative grouping of 486 pseudomonas strains by serology and pyocine typing is shown in Table 6 Some of the serogroups appear to contain a predominance of certain pyocine types The frequency distribution of the pyocine types within each of the sero groups can be distinguished from that for the whole collection It is notable, that two thirds of the O 2 *strains* belong to pyocine type 1 (p1) 48/88 strains of O 3 are p5, 34/50 of O 5 are p1, 56/85 O 6 *strains* are p1 The non agglutinating and the self agglutinable isolates were 11/30 and 13/36 of p1 respectively These relationships are significant

If the population is considered from the point of view of the pyocine types p3 and p5 are notable for serogroup predominance Thus 56/97 p3 strains belong to O 6 and 48/66 of p5 to O 3

DISCUSSION

With the rising frequency of pseudomonas infections the increasing numbers of patients with weakened resistance to infection and the nosocomial nature of pseudomonas infections there is a need for a dependable typing procedure for *P aeruginosa*

Serological investigations on *P aeruginosa* has been carried out since the turn of the century, but the major importance of all these works appears only to be the demonstration of antigenic heterogeneity within the species (4) The breakthrough in subdivision by O antigens came with the works of *Habs* (10) which was followed up by *Kleinmaier* (14) *Sandvik* developed a parallel schema for cattle and showed how the groups I–VII compared with the Habs group 1–12 No counterpart was found for O II, which has been adopted in the Habs schema as O 13 (21 29) The Sandvik system has subsequently been supplemented by a group O VIII (25) which has presently been identified as Habs O 8 *Kleinmaier* & *Muller* (15) demonstrated that precipitation reac

tions confirmed the typing schema of Habs as compared with the agglutination of heated cells

Presently, the slide agglutination procedure to live cells has been used in conformity with previous usage (14 16, 23, 24, 29) *Lanyi* (16, 17) observed better agglutination with live antigens during his extensive methodological research and interpreted this to indicate that pseudomonas did not possess antigens which mask the O antigens *Meitert* & *Meitert* (19) found slide agglutination to render 1–2 steps lower titres than agglutination with the same antigens *in tubes*, but, like *Kleinmaier* (14) and *Lanyi* (16), found the slide procedure with live cells just as specific and reliable as the tube procedure *Sandvik*, for strains isolated from animals also employed slide agglutination (23, 24)

Self agglutination was noted for as much as 7 4 per cent of the strains presently examined This phenomenon appears to be a difficult one also for other workers (12, 16, 18, 23 29), although attempts have been made to avoid it by modifying cultivating conditions *Mikkelsen* (20) thus observed 12 per cent self agglutinable strains.

Unfortunately, the work of *Habs* in establishing a serogrouping schema for human pseudomonas strains has been duplicated at other centers (16, 18, 19, 27) where different serogrouping sets have been developed without comparison with Habs antigens In presenting his set for strains from cattle, *Sandvik* (23, 24) felt the necessity to correlate the cattle serogroups to the human (*i e Habs*) counterparts Less confusion would certainly have evolved if the efforts of the subsequent works had centered upon further elucidation of the existing schemas *Verder* & *Evans* (27) in 1961, and *Lanyi* (16) in 1966/1967 present ed schemas which later (17) in addition to O groups also included H antigen typing *Lanyi* presented convincing evidence that pseudomonas lacked thermolabile somatic antigens and that the thermolabile antigens which are demonstrable by agglutination are located on the flagella (17)) *Verder* & *Evans* identified 10 groups *Lanyi* 13 O groups which appeared to render a good separation between strains *Meitert* (18) presented a system with 10 groups, but the strains on which this was based must have been fairly related, since 51 per cent belonged to one single serogroup

The discrepancies found for the various reference strains in different laboratories (Table 2) are remarkable These would seem to indicate that the antigenic structure of *P aeruginosa* is not constant The differences, however, may be caused by the antigen response in the individual rabbit, and differences *in immunization technique or schedule* (20) Individual reference sub strains may vary as is illustrated by my O 11 which reacted with more factor sera than in any other laboratory while at the same time its serum rendered more heterologous reactions than usual It is notable that both *Wahba s* and my sera were obtained by the same immunization schedule, since his sera and mine demonstrate somewhat more cross reactivity than has been noted by most others An obvious source of variation may derive from the interpretation of weak agglutination reactions

It is of considerable relevance that *Henriksen* (11) has found similar interlaboratory discrepancies in the reported crossreactivity within a set of reference strains of *Klebsiella* Thus, the variability found for *P aeruginosa* does scarcely imply special conditions for this species

One of the purposes of this communication was to compare some of the different pseudomonas serogrouping systems The Lanyi reference strains (16) were tested with Habs sera Some of the Lanyi strains were either self agglutinable or did not react with the Habs sera, but most of the strains were classifiable in one of Habs groups There were several representatives of some Habs groups, *e g* O 6 It is interesting that the Lanyi specificity 5d is a distinct property of Habs O 8, whereas the cross reacting Habs O 7 and O 8 share the Lanyi antigens 5ab

The present recognition of the Wahba O 14 may be controversial since it is so much alike the Habs O·6 Others have considered

the two indistinguishable However, the present absorption experiments demonstrate a distinction between the two Whether this is sufficient for a recognition of a new group may be a matter of opinion An important feature to decide the matter was that without a distinction, group O 6 would have covered 27 per cent of the strains This would have rendered an insufficient subdivision in an epidemiological context

The recognition of the Wahba O 14 has been done in accordance with previous usage (28, 29), but may cause some confusion, since an international collaborative group under the auspices of the Subcommittee on Pseudomonas and Related Organisms in July, 1971, suggested another strain of different specifities as a candidate for O 14 (*Liu*, personal communication) *Mikkelsen* (21) has recently proposed yet another strain as O 14

Since previous results with pyocine typing had been discouraging in terms of reproducibility (1), the finding made presently that the pyocine typing procedure of *Gillies* & *Govan* (7) was satisfactory is important The main objection to pyocine typing is that it usually renders only insufficient discrimination for epidemiological purposes Below, major pyocine types are listed as designated by the authors in question with the percentages of occurrence appearing within parentheses

Darrell & *Wahba* (ref 6) B (37 7), D (16 7), A (11 7), P (6 2), L (5 6)
Zabransky & *Day* (ref 30) A (30 9), D (14 1), O (3 1), F (2 9), I (1 6)
Gillies & *Govan* (ref 7) 1 (34 2), 3 (25 2) 5 (5 7), 10 (2 9), 35 (2 9)
Bergan (Table 6) 1 (31 7), 3 (20 0), 5 (13 6) 10 (6 4), 11 (2 1)

In view of the lumping of strains obtained by pyocine typing *Govan* & *Gillies* (9) more recently have reported a procedure for the subdivision of p1 However this also renders 3 major groups accounting for a total of approximately 3 4 of the strains and with 17 4–30 3 per cent of the strains each In effect by this extra typing step, only two more groups are obtained in addition to aid in the subdivision of the group comprising 70 per cent of the pseudomonas strains

Thus, pyocine typing in the *Govan* (8) and *Gillies* & *Govan* (7) alternative emerges as a procedure which is easy to handle even with limited resources, is reproducible and renders epidemiologically relevant information However, the procedure has such a limited capacity for differentiating strains, that it is not sufficient as an epidemiological marker by itself, unless the organism belongs to a rare pyocine type such as in the investigation by *Tinne et al* (26) where pyocine typing has been used for a succesful elucidation of a nosocomial problem

A different situation may exist for serogrouping The discrimination rendered by the Habs system was such that the groups 3, 5, and 6 contributed approximately half of the strains Particularly O 6 dominated, *Habs* (10) noted this group in 32 9 per cent, *Wahba* (29) in 34 2, *Verder* & *Evans* (27) the corresponding serogroup O 2 in 27 2, and *Mikkelsen* (21) in 16 3 per cent In animals, the corresponding serogroup occurred in 26 2 per cent (25)

The relationship between serogroup distribution and pyocine types shown in Table 6 indicates a correlation between certain pyocine types and serogroups The same finding was made by *Wahba* (28) who combined the Habs serogrouping scheme with another pyocine typing procedure (28, 29), and by *Csiszar* & *Lanyi* (5) One should, however, be very cautious, such data do not necessarily indicate a firm biological phenomenon This is particularly important since the entities where enough elements were present to allow a reasonable estimate are the very same entities which occur most frequently in the whole material It may look overwhelming or 56/85 O 6 strains belong to p3, and 56/97 p3 strains are O 6, but at the same time O 6 and p3 are most frequent, each contributing some 20 per cent of the strains That is to say, however, that if only the laws of probability were followed and serospecificity and pyocine production were stochastic elements their conjoint occurrence should only occur in 4

per cent of the cases Consider, on the other hand, the most frequent sero and pyocine entities O 6 and p1 Only 6/85 of the O 6 strains are p1, and only 6/154 of the p1 strains are O 6 Consequently, there appears to be a true relationship between some serogroups and pyocine types

It is interesting to note that *Homma & Suzuki* (13) found that pyocine activity was associated with the protein component of the lipopolysaccharide protein complex of the cell wall, the O antigenicity of *P aeruginosa* has also been associated with this complex, although the lipopolysaccharide component probably is the major antigenic determinant

Consequently, pyocine typing and serogrouping seem interrelated in some way What consequences this might have for a combined use on clinical isolates will be further elucidated (3)

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EPIDEMIOLOGICAL MARKERS FOR *PSEUDOMONAS AERUGINOSA*

2 Relationship Between Bacteriophage Susceptibility and Serogroup or Pyocine Type

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The mutual interrelationship between bacteriophage susceptibility of *Pseudomonas aeruginosa* and serogroup and pyocine type of the bacterial strains have been investigated A possible interrelationship has been found only for a few phages to the effect that the phages may have preference for certain serogroups Bacteria of the pyocine type 5 tended to be lysed more efficiently by high avidity phages than other pyocine types Only one of the 113 phages seemed to have any preference for a given pyocine type There was no tendency for given serogroups or pyocine types on the other hand to be lysed more frequently by certain phages than would be expected from the avidity of the phages on the entire strain collection

It has previously been contended that there is an interdependence between certain bacteriophage types of *Pseudomonas aeruginosa* and particular serogroups (5) Evidence has been presented to show that lysogenization in *P aeruginosa* may cause change in bacterial O antigen (11) For salmonella it has been considered that small variation in antigens be responsible for alterations in susceptibility to phage (8) In *Staphylococcus aureus*, it has been an axiom that phages of particular phage groups tend to lyse predominantly strains of a given Cowan serogroup (6 12), this was studied, however, by *Oeding & Williams* (12), who found that there was no firm interdependence between bacterial serotype and phage susceptibility

Consequently it would be of considerable interest to find out whether the three available typing approaches for *P aeruginosa* are interrelated Indication of a partial correspondence in serogroup and pyocine type has been presented (4)

In this communication will be studied whether pseudomonas phages preferentially lyse bacteria of given serogroups or pyocine types The inverse, whether the bacteria of a particular serological or pyocine entity are lysed selectively by certain phages will also be investigated

MATERIALS AND METHODS

Bacterial Strains and Bacteriophages

Subject to study have been 486 bacterial strains and 113 pseudomonas bacteriophages. The phages have mostly been derived from previous phage typing sets the bacteria partly from this hospital and partly from abroad A more detailed description of bacteria and phages is found in a previous communication (2)

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TABLE 1 *Distribution (in Per Cent) of Serogroups within Groups of Bact*

Serogroup	Bacteriophages											
	12	17	39	43	45	54	68	71	74	78	8…
NT*		3	4	5	5	6	2			7	
SA§		6	6			9	1	9	7	7	
1		6	9	2		5	10	12	11	7	
2		3	8	5	9	3	1	0	4	6	
3	17	63	11	44	5	56	12	18	15	13	1
5		*3*	*10*	*20*	*18*		*9*	*9*	*11*	*5*	
6	71	3	24	2	14	10	35	21	18	22	2
9			6	13	18	5	5	9	11	6	1
14			8		18		9	12	7	7	
Per cent bacteria lysed	36	7	23	8	5	17	29	7	6	25	2

* NT = Non typable § SA = Self agglutinable

Typing Procedures

The procedures for phage typing serogrouping and pyocine typing have been detailed previously (2, 4)

RESULTS

1 Presentation of Data

Due to the character and amount of data, only part of the results shall be presented As examples the findings pertaining to the phages with the highest percentage of lysis, the phage typing system of *Lindberg et al* (10) (which has received the most wide spread recognition) and the new phage typ ing system developed by objective procedures (3) would seem to be of the most interest The first set of phages lyses such a large number of pseudomonas strains that reason ably safe conclusions may be drawn and the two last phage typing sets have been chosen for the reason that documentation in relation to these is particularly desirable

TABLE 2 *Distribution (in Per Cent) of Serogroups within Groups of Bacteria Lysed by the Same Bacteriophage Data Given for the Phage Typing Set of* Lindberg et al *(10)*

Serogroup	Bacteriophages																Total number (and %) within each serogroup
	1	2	4	5	7	8	10	11	12	13	14	15	16	17	18	20	
NT*	4	3	2	6	5	8	0 8	4		0 9	4	3	2	3	3	4	30 (6)
SA§	4	5	4	6		10	8	5		10	2	8	6	6	7	4	36 (7)
1	2	10	2	6	13	2	5	8		7	6	16	5	6	7	13	27 (6)
2	1	[illegible]	6	7		1	7	8		6	7	3	8	3	7		30 (6)
3	35	3	6	27	28	52	14	13	17	10	20	27	16	63	14	15	88 (18)
5	3		8	11	3	1	7	11		12	8	4	9	3	12	4	50 (10)
6	27	3	29	12	18	13	27	20	71	22	23	16	26	3	18	21	85 (18)
9	9	11	16	7	12	3	16	12		15	10	5	12		16	16	30 (6)
14	7		9	8	8	2	2	7		5	5	4	3		4	16	27 (6)
Per cent bacteria lysed	37	39		10	12	19	27	36	5	23	26	16	35	7	25	11	

* NT = Non typable § = Self agglutinable

	88	95	102	104	110	111	Auxiliary phages 6	11	22	64	75	Total number (and %) within each serogroup
	6		7	2	5	12	6	4	6	0 9	8	30 (6)
	11	14	9	11	7	20	7	5	5	3	12	36 (7)
	11	9	6	8	7	4	5	8	5		14	27 (6)
	6	11	4	2	7	12	6	8		10	2	30 (6)
	18	20	17	31	14	4	27	13	40	17	28	88 (18)
	6	14	15	3	14	12	10	11	2	13	2	50 (10)
	11	11	9	9	5	12	13	20	17	26	4	85 (18)
	11		6	12	12		7	12	14	16	12	30 (6)
	4	3	9	5	5		8	7	2	2	5	27 (6)
	16	7	14	22	9	5	44	36	26	23	19	

2 *Concordance between Bacteriophage Susceptibility and Serogroups*

2 1 *Distribution of Serogroups within Groups of Bacteria lysed by the Same Phage*

The Tables 1, 2, and 3 indicate how strains susceptible to each phage are distributed between the different serogroups The percentages in the body of the Tables have been evaluated by comparing with the percentage distribution of serogroups for the whole bacterial collection as it appears in the column to the far right In the Tables, the figures which may indicate overrepresentations have been underlined

Only items where the sums of rows and columns each carry 5 per cent or more have been considered, and mostly only frequencies close to or above the double of the percentage in the right hand column have been in

TABLE 3 *Distribution (in Per Cent) of Serogroups within Groups of Bacteria Lysed by the Same Bacteriophage Data Given for Phages with the Highest Percentages of Lysis*

Serogroup	Bacteriophages 1	2	6	11	16	23	32	34	37	61	63	66	72	93	107	109	Total number (and %) within each serogroup
NT*	4	3	6	4	2	3	5	7	5	5	4	6	4	8	4	6	30 (6)
SA§	4	5	7	5	6	6	5	7	5	8	6	7	4	9	7	7	36 (7)
1	2	10	5	8	5	8	5	6	8	8	7	8	7	6	4	7	27 (6)
2	1	2	6	8	8	6	7	4	5	5	1	5	1	5	3	4	30 (6)
3	35	13	27	13	16	20	35	28	28	12	34	13	35	20	22	24	88 (18)
5	3	7	10	11	9	10	11	10	10	8	0 6	8	0 6	7	3	3	50 (10)
6	27	32	13	20	26	23	15	17	14	22	18	20	18	17	22	21	85 (18)
9	9	10	7	12	12	12	6	6	6	8	13	8	11	10	7	6	30 (6)
14	7	7	8	7	3	4	5	6	8	6	6	7	7	6	7	6	27 (6)
Per cent bacteria lysed	37	39	44	36	35	30	26	34	40	32	34	32	32	32	33	32	

* NT Non typable § SA = Self-agglutinable

6*

TABLE 4 *Distribution (in Per Cent) of Phage Susceptib*

Serogroup	Bacteriophages											
	12	17	39	43	45	54	68	71	74	78	82	8
NT*		3	13	7	3	17	10			27	7	1
SA§		6	19			19	19	8	6	22	19	2
1		7	37	4		15	52	15	11	30	22	3
2		3	30	7	7	7	7		3	23	20	1
3	5	23	14	21	1	51	19	7	5	17	18	1
5		2	22	16	8		24	6	6	12	16	1
6	20	1	31	1	4	9	58	8	6	32	26	1
9			23	17	13	13	23	10	10	23	60	3
14			33		15		4	15	7	30	4	1
Per cent bacteria lysed	36	7	23	8	5	17	29	7	6	25	21	1

* NT = Non typable, § SA – Self agglutinable

dicated Significantly lower figures have only been indicated in a few instances

2 2 Distribution on Phage Susceptibility for Strains Belonging to the Same Serogroup

The Tables 4, 5, 6 show how strains of the same serogroup are lysed by the various phages In Table 6 are indicated the phages with the highest lytic activity For a differentiation of deviations in phage susceptibility, the percentage figures must be compared with the values in the bottom line

3 Concordance between Bacteriophage Susceptibility and Pyocine Type

3 1 Distribution on Pyocine Types of Strains with Susceptibility to the Same Phage

The Tables 7, 8, and 9 indicat how the strains lysed by a given phage are distributed

TABLE 5 *Distribution (in Per Cent) of Phage Susceptibility within Serogroups Data Given for the Phage Typing Set of* Lindberg et al *(10)*

ogroup	Bacteriophages																	Total number (and %) within each serogroup
	1	2	4	5	7	8	10	11	12	13	14	15	16	17	18	19	20	
*	23	17	3	40	10	23	3	23		3	17	7	13	3	10		7	30 (6)
§	19	28	6	31		25	28	25		31	6	17	31	6	22		6	36 (7)
	11	67	4	41	30	7	22	48		30	26	48	30	7	33		26	27 (6)
	7	13	10	47		3	30	67		23	30	7	43	3	30			30 (6)
	72	27	3	59	19	53	21	25	5	13	28	24	32	23	19		9	88 (18)
	12	26	8	42	4	2	18	38		26	20	6	32	2	30		4	50 (10)
	58	70	17	27	13	14	41	41	20	28	34	15	52	1	26		13	85 (18)
	53	60	27	47	23	10	67	70		53	40	13	70		67		27	30 (6)
	44	52	19	56	19	7	7	44		19	22	11	19		19		30	27 (6)
cent teria lysed	37	39	10	40	12	19	27	36	5	23	26	16	35	7	25		11	

* NT = Non typable § SA – Self agglutinable

ithin Serogroups Data given for the New Phage Typing Set (3)

95	102	104	110	111	Auxiliary phages 6	11	22	64	75	Total number (and %) within each serogroup
	17	7	7	10	43	23	23	3	23	30 (6)
14	17	33	8	14	42	25	17	8	31	36 (7)
11	15	30	11	4	37	48	22		48	27 (6)
13	10	7	10	10	43	47	3	37	7	30 (6)
8	14	38	7	1	65	25	57	22	30	88 (18)
10	20	6	12	6	44	38	4	30	4	50 (10)
5	7	12	2	4	32	41	25	34	5	85 (18)
	13	43	17		50	70	57	60	37	30 (6)
4	22	19	7		63	44	11	7	19	27 (6)
7	14	22	9	5	44	36	26	23	19	

between pyocine types Frequencies which are approximately doubled relative to the right hand column have been underlined, only in stances where the frequencies for rows or columns were above 5 per cent have been entered in the Tables Only two of the items entered in Table 9 concerning the phages with high percentage of lysis have been noted as somewhat high Of the strains lysed by the new set, those lysed by the phage 12 belong more often to pyocine types 3 and 5 than would be expected The phage 17 primarily (2/3) lyses pyocine type 5 (p5), about half of the bacterial strains lysed by phage 54 are p5, and about 2/3 of the strains lysed by phage 111 belong to p1 The set of Lindberg et al shows interesting features with the phages 1, 8 and 17

TABLE 6 *Distribution (in Per Cent) of Phage Susceptibility within Serogroups Data Given for the Phages with the Highest Percentages of Lysis*

Serogroup	Bacteriophages 1	2	6	11	16	23	32	34	37	61	63	66	72	93	107	109	Total number (and %) within each serogroup
NT*	23	17	43	23	13	17	20	37	33	23	20	30	20	43	23	30	30 (6)
SA§	19	28	42	25	31	22	17	33	25	33	25	31	17	39	31	28	36 (7)
1	11	67	37	48	30	44	22	33	56	48	44	48	41	33	26	37	27 (6)
2	7	13	43	47	43	27	27	20	33	27	8	27	7	27	17	20	30 (6)
3	72	27	65	25	32	33	49	52	60	21	64	22	61	35	40	42	88 (18)
5	12	26	44	38	32	28	28	34	40	24	2	26	2	22	10	10	50 (10)
6	58	71	32	41	52	39	21	33	31	39	35	35	33	31	41	38	85 (18)
9	53	60	50	70	70	57	23	33	37	43	73	43	57	50	37	30	30 (6)
14	44	52	63	44	19	22	22	33	56	37	33	37	37	33	41	33	27 (6)
Per cent bacteria lysed	37	39	44	36	35	30	26	34	40	32	34	32	32	32	33	32	

* NT = Non typable § SA = Self agglutinable

TABLE 7 *Distribution (in Per Cent) on Pyocine Types of Strains with Susceptibility to the Sam[e] Bacteriophage Data Given for the New Phage Typing Set (3)*

[Py]ocine [ty]pe	Bacteriophages 12	17	39	43	45	54	68	71	74	78	82	88	95	102	104	Auxiliary phages 110	111	6	11	22	64	75	Perc[entage] of ba[cteria] with[in each] pyoci[ne type]
	4	16	31	37	41	14	20	33	25	29	41	33	46	32	28	40	64	33	35	20	42	23	
	79	6	28	5	9	9	35	24	18	24	23	15	11	15	11	23	8	18	19	21	27	8	
	42	66	3	29		58	8	3	4	9	10	13	11	9	26	7	4	19	6	32	6	21	
		3	4	25	9	1	3		11	6	1		9	9	6	7		2	5	2	2	4	
[Pe]r cent [ba]cteria [lys]ed	36	7	23	8	5	17	29	7	6	25	21	16	7	14	22	9	5	44	36	26	23	19	

TABLE 8 *Distribution (in Per Cent) on Pyocine Types of Strains with Susceptibility to the Same Bacteriophage Data Given for the Phage Typing Set of* Lindberg et al *(10)*

Pyocine type	Bacteriophages 1	2	4	5	7	8	10	11	12	13	14	15	16	17	18	20	Percentage of bacteria within each pyocine type
1	17	24	37	34	23	8	40	35	4	41	32	18	39	16	40	25	32
3	23	31	27	16	20	17	26	19	9	20	25	19	24	6	18	25	20
5	31	7	6	20	20	51	9	6	4	8	14	11	10	66	7	4	14
10	1	3	2	2	3	3	0 8	3	6	0 9	5		0 6	3	4	2	6
Per cent bacteria lysed	37	39	10	40	12	19	27	36	5	23	26	16	35	7	25	11	

TABLE 9 *Distribution (in Per Cent) on Pyocine Types of Strains with Susceptibility to the Same Bacteriophage Data Given for the Phages with the Highest Percentage of Lysis*

Pyocine type	Bacteriophages 1	2	6	11	16	23	32	34	37	61	63	66	72	93	107	109	Percentage of bacteria within each pyocine type
1	17	24	33	35	39	32	29	24	32	41	20	38	19	30	23	21	32
3	23	31	18	20	24	21	11	16	13	24	19	23	20	18	26	26	20
5	31	7	19	6	10	12	27	24	21	5	30	8	31	13	18	25	14
10	1	3	2	5	0 6	3	2	3	2	5	1	5	1	2	6	5	6
Per cent bacteria lysed	37	39	44	36	35	30	26	34	40	32	34	32	32	32	33	32	

3 2 *Distribution of Phage Susceptibility among Strains of Identical Pyocine Groups*

The Tables 10 11 and 12 show whether there is any tendency for strains within given pyocine types to be lysed by bacteriophages in a way which deviates from the whole collection of bacteria High incidences are indicated by underlining as above It is notable in Table 12 that the p5 strains are lysed by the high avidity phages to an extent which exceeds the level found for the entire bacterial population Most notable is the finding that every one of these strains are lysed by the phage number 93

The results for the new phage set and the

Lindberg et al set also indicate that the p5 strains are more susceptible to individual bacteriophages

4 Typing of Reference Strains for the Typing Procedures

It was considered that knowledge of the epidemiological types of the reference strains for serogrouping indicator strains for pyocine typing and host propagating strains for the new phage typing procedure could shed some light on the topic of interdependence between phage susceptibility and sero and pyocine typing characteristics

Table 13 shows the serogroup pyocine type and phage susceptibility patterns of the reference strains for the new phage procedure

Table 2 when compared with the results on serospecificity for the host propagating strains reveal that there is a predominant lysis of strains of the same serogroup as the propagating strain only for the four phages 12 43 82 and 111 No further interrelationship between phage susceptibility pyocine type or serospecificity is discernible from the typing patterns of the reference strains compared with the Tables 1–12

DISCUSSION

It would seem desirable to apply objective procedures for evaluating structure in the (n×m) matrices shown in Tables 1 12 Unfortunately no readily available statistical procedure could be identified which was suitable for the purpose* Simple t test or Chi square tests for 2×2 tables are not applicable as they would forfeit the multicomponent structure of the items

One of the most important questions which one wanted to elucidate was the question of whether certain phages tended to lyse predominantly bacteria which belonged to one or a few serogroups. The data for the phages with high lytic activity seem to indicate that no firm relationship exists between the two Still it would seem that some phages are semi discriminatory with more frequent lysis of given serogroups Such a relationship is more pronounced for the new phage set than for the set of Lindberg et al (10) For instance, 2/3 of the strains lysed by the phage 12 belong to serogroup 6 Approximately the same frequency is found for group 3 within strains lysed by phage 17 The phage 54 is another discriminatory phage Except for these few notable instances the interrelationship of phage lysis and serogroup specificity is but feebly pronounced for the *Pseudomonas aeruginosa* strains. This is in accordance with the previous communication on this point (1)

Within the phages for *Staphylococcus aureus* a predominance of bacteria within one of the Cowan serogroups has been contended for each of the phages in the international typing set This has even led to the organization of the staphylococcal phages in groups of phages which tend to lyse the same groups of strains *Oeding & Williams* (12) however established that there were frequent aberrations from this orderly schema for the staphylococci

Comparison of data of Table 2 and Table 13 allows further elucidation on the question of serogroup predominance of the strains lysed by certain pseudomonas phages It was found that the serogroups of the phage host propagating strains rarely are repeated as the most frequent group within the strains lysed by each phage Only in four instances could such an increased frequency be detected

In the Tables 1 12 the figures which are higher than approximately twice the frequency distribution for the entire bacterial collection have been specially indicated to facilitate interpretation of the data Since it is considered that positive traits are of higher predictive value than negative ones and a frequency below the expected might be due to a redistribution with predominance of certain groups less weight has purposely been put on underrepresentations of certain entries To

* In this connection the Norsk Regnesentral was consulted.

TABLE 10 *Distribution (in Per Cent) within Each Pyocine Ty*

Pyocine type	Bacteriophages										
	12	17	39	43	45	54	68	71	74	78	82
1	6	3	22	10	6	7	18	7	5	23	27
3	20	2	32	2	2	7	51	8	5	30	24
5	2	**32**	5	**18**		**71**	17	2	2	17	15
10		3	13	3	7	3	13		10	23	3
Per cent bacteria lysed	36	7	23	8	5	17	29	7	6	25	21

TABLE 11 *Distribution (in Per Cent) within each Pyocine Type of Bacteriophage Susceptibility Data Given for the* Lindberg et al *(10) Phage Typing Set*

Pyocine type	Bacteriophages																Percentage of bacteria within each pyocine type
	1	2	4	5	7	8	10	11	12	13	14	15	16	17	18	20	
1	20	30	12	42	9	5	33	40	0 6	30	26	9	44	3	32	8	32
3	43	61	13	32	12	16	35	34	20	23	33	16	43	2	23	13	20
5	**83**	21	5	61	18	70	17	17	2	14	27	14	26	32	12	3	14
10	7	19	3	13	7	10	3	26		3	19		3	3	16	3	6
Per cent bacteria lysed	37	39	10	40	12	19	27	36	5	23	26	16	35	7	25	11	

TABLE 12 *Distribution (in Per Cent) within each Pyocine Bacteriophage Susceptibility Data Given for Phages with Highest Percentage of Lysis*

Pyocine types	Bacteriophages																Percentage of bacteria within each pyocine type
	1	2	6	11	16	23	32	34	37	61	63	66	72	93	107	109	
1	20	30	46	40	44	30	23	25	40	42	21	39	19	31	24	21	32
3	43	61	40	34	43	31	14	38	25	38	32	37	32	32	42	41	20
5	**83**	21	61	17	26	26	**52**	59	62	12	**74**	18	**71**	**100**	42	59	14
10	7	19	13	26	3	13	10	16	10	23	7	23	7	10	29	23	6
Per cent bacteria lysed	*37*	*39*	*44*	*36*	*35*	*30*	*26*	*34*	*40*	*32*	*34*	*32*	*32*	*32*	*33*	*32*	

avoid erroneous interpretation from chance variability, only frequency data have been considered where both the sums of rows and columns were 5 per cent or above

The tendency for strains within a given serogroup to be lysed more often by certain phages is small The instances where such a relationship, *i e* overrepresentation is found are so few that one should refrain from suggesting that there is a true relationship

This corresponds to the conclusion drawn for *S aureus* by *Oeding & Williams (12)* who stated that "very little correlation was seen between the individual types recognized by the two methods " Still, the tendency for given phages to predominantly lyse bacteria of given serological specificities is clearly more pronounced for the staphylococci than

Bacteriophage Susceptibility Data Given for the New Phage Typing Set (3)

95	102	104	110	111	Auxiliary phages 6	11	22	64	75	Percentage of bacteria within each pyocine type
10	14	19	11	10	46	39	16	31	14	32
6	10	12	10	2	40	34	27	31	7	20
	9	42	5	2	61	17	61	11	29	14
10	19	19	10		13	26	7	7	13	6
7	14	22	9	5	44	36	26	23	19	

TABLE 13 *Serogroup, Pyocine Type and Bacteriophage Susceptibility of Host Propagating Strains for the New Pseudomonas Phage Typing Procedure (3)*

Strains	Serogroup	Pyocine type	Phage susceptibility
1	6	3	1,2
2	SA*	38	2,11
3	SA	1	3,6,7,12,14,15,18/4
4	6,14	1	3,4,5,6,7,8,11,12 15/1
5	5	1	5
6	10	1	3,4,5,6,7,8 10,11,12,15
7	2	1	5,6 7,8,12/16,17
8	9	3	8,12
9	6	3	9,12,16,17
10	5	1	4,5,6,8,10
11	5	1	Aux 2
12	9	1	8,12/10 15
13	6	3	8 13,19/3,16
14	2	10	14
15	5	1	2,5 8 12,14,15
16	13	3	9,12,16
17	2	10	12
18	6,14	17	8 12,14,15,18
19	11	1	3 6,12,14,15,16 19
Aux 1	2 5	1	2,3,5,7,10,14,15,19
Aux 2	3	64	12
Aux 3	6	3	9,12
Aux 4	6	3	1,8,9,10 12,14,15,18/4
Aux 5	6	3	8,12

* SA = Self agglutinable

for pseudomonas. In *S aureus*, some 80 per cent of the strains lysed by the phages of group I belong to serogroup 1, 50 per cent of the strains sensitive to group II phages belong to serogroup 2, and some 80 per cent of the strains lysed by group III phages are alotted to the serogroups 2 and 3 (12)

An attempt to lump different pseudomonas serospecificities together to establish a simple differentiating structure like the one approached in the staphylococci was entirely unsuccessful Even cross reacting pseudomonas serospecificities did not behave in the same way in relation to phage susceptibility

It may seem surprising that the tendency for phages to lyse bacteria of given sero-

groups is not more pronounced than it has turned out to be Both O-antigens and phage attack are determined by surface structures on the bacterial cell Differences in phage receptor site configurations could conceivably render altered antigenic specificity The present results may be interpreted to indicate that the determinative units for antigenicity and the phage receptor overlap It could also be that fimbriae play a role in the absorption of phages as has been found in enterobacterial, such a difference in topography of O antigens and phage receptors would adequately account for the findings made

The instances where the distribution of pyocine types deviate from the collective frequency figures for the whole collection are so few that one must be particularly careful in interpreting these results to indicate that any preference exists Only in a few distances do the results invite the conclusion that some phages have preference for certain pyocine types Phage 93, for instance, only lyses 32 per cent of all strains examined but all p5 strains are lysed by it Bacteria of p5 seem to be more susceptible to lysis by high avidity phages The finding that there is mostly no relationship between phage susceptibility and pyocine type is not surprising in view of the fact that the latter is a test for the host spectrum of an agent produced by the bacterium which is typed Only if the pyocines were in a juxtaposition to surface structures of the producer cell before liberation from the producer cell could a relationship between pyocine type and phage susceptibility be understandable

Some relationship, however, seems to exist between the host spectra of the pyocines and pseudomonas phages *Homma & Shionoya* (7) found that antisera to bacteriophages inhibited the activity of pyocines They also found that phage activity was neutralized by antisera against pyocines The latter finding was verified by *Ito & Kageyama* (9), who also observed that sensitivity to certain phages was followed by sensitivity to given pyocines Indeed, *Ito & Kageyama* from their studies which were supplemented by electron microscopy concluded that phage and pyocine receptors were identical

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EPIDEMIOLOGICAL MARKERS FOR *PSEUDOMONAS AERUGINOSA*

3 Comparison of Bacteriophage Typing, Serogrouping, and Pyocine Typing on a Heterogeneous Clinical Material

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On epidemiologically heterogeneous pseudomonas strains have been compared the use of bacteriophage typing by two procedures serogrouping and pyocine typing The new phage typing procedure of the author was demonstrated to be preferable to a previous phage typing procedure which is extensively used A combination of serogrouping and pyocine typing renders a good alternative typing procedure to phage typing for the bacteriological laboratories with a relatively modest load of *Pseudomonas aeruginosa* Phage typing is preferable to other typing procedures in terms of rendering a high degree of differentiation and demonstrating strain relatednes in serial cultures from the same sites

The three available typing procedures for *Pseudomonas aeruginosa* have been the subject of several reports (see Table 1 and references (6, 7)) Usually, one typing procedure has been used alone (2, 10, 11, 13, 15, 35 39, 42, 43), but occasionally more than one procedure have been used simultaneously (1, 12, 14, 16, 18, 26, 41) All three methods have only been used in combination once previously (29)

In this communication, the concomitant use of the Habs serogrouping system (6), pyocine typing technique of *Gillies & Govan* (15), the new phage typing procedure of the author (2 3), and the widely distributed phage typing set of *Lindberg et al* (24, 25) are to be compared The parallel use of the two phage typing methods allows a comparison between them and an evaluation of their combined use in nosocomial strains

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MATERIALS AND METHODS

This investigation has been performed with 284 strains of *Pseudomonas aeruginosa* collected during a three year period (1965-1968) from the regular routine of the Institute of Bacteriology, Rikshospitalet Oslo The species diagnosis was ascertained according to the criteria indicated previously (2)

The typing procedures have been elaborated in previous papers (2, 6)

RESULTS

1 Source of Infection

1 1 Organ Origin

The clinical sources of the pseudomonas strains are indicated in Table 2 Urine ulcers and wounds, and the respiratory tract were the three most frequent categories.

1.2 Hospital Sites

In Table 3 are listed the numbers of positive specimens from each department. The

TABLE 1 *Reported Portions of Typed Pseudomonas Strains by Pyocine Typing, Serogroupin Bacteriophage Typing in Different Investigations*

Pyocine typed Authors	%	Serogrouped Authors	%	Phage typed Authors
Darrell & Wahba (1964)W	92 4	*Sandvik* (1960)	94 3	*Gould & McLeod* (1960
Wahba (1965)W	87 8	*Chia-Ying* (1963)	96 0	*Hoff & Drake* (1960)
Gillies & Govan (1966)G	88 4	*Meitert* (1964)	84 6	*Pavlatou & Kaklamani* (
Sjöberg & Lindberg (1967)W	87 7	*Wahba* (1965)H	99 7	*Postic & Finland* (1961)
Matsumoto et al (1968)W	81 0	*Murashi et al* (1966)VE*	84 0	*Lindberg et al* (1964)
Govan & Gillies (1969)G	92 4	*Matsumoto et al* (1968)VE*	93 0	*Meitert* (1965)
Zabransky & Day (1969)W	93 2	*Lanyi* (1970)*	99 3	*Sutter et al* (1965)
Bodey (1970)G	69 0	*Mikkelsen* (1970)H	74 6	*Sjoberg & Lindberg* (19
Booth (1970)G	87 6			
*Own results*G	96 7	Own resultsH	86 4	Own results

W = *Wahba*'s pyocine typing method (41) used, G = *Gillies & Govan*'s pyocine typing method used, VE = *Verder & Evans*'s (40) serogrouping schema used, H = *Habs*'s serogrouping system 41), * = percentage calculated only for O groupable strains

TABLE 2 *Distribution of Strains upon Clinical Sources*

Clinical source	Year 1965	1966	1967	1968	Tot
Urine	17	23	16	17	73
Ulcer/wound	14	9	11	8	42
Tracheal secretion/expectorate	14	9	10	8	41
Ear	2	3	5	7	17
Feces	1	2	1	0	4
Vagina/pleural cavity/cerebrospinal fluid	0	0	0	3	3
Total	48	46	43	43	180

Each patient has been counted once, unless more sites in the same patient are found

TABLE 3 *Distribution of Psodomonas Strains upon Hospital Sites*

Hospital source (department)	Year 1965	1966	1967	1968	Tota
Surgery (A + B)*	16	12	17	12	57
Pediatrics	9	10	7	6	32
Oto rhino-laryngology	5	4	5	9	23
Neurology	7	6	4	4	21
Medicine (A + B)*	2	3	5	4	14
Dermatology	4	3	4	2	13
Pulmonary	2	1	1	3	7
Out patients	0	4	0	0	4
Neurosurgery	0	1	0	2	3
Obstet/GYN	1	1	0	1	3
Pathology	1	1	0	0	2
Ophtalmology	1	0	0	0	1
Total	48	46	43	43	180

Each patient has been counted once, unless more sites were found in the same patient
* There are two departments of surgery and of medicine, each signified A or B

frequencies indicated probably do not show the true incidence in each department, since they to some extent reflect the diligence with which the clinical departments take samples for bacteriological examinations Nevertheless, it is notable that the most important sources have been the surgical departments (general surgery and oto rhino laryngology) and the pediatric department Urinary tract infection in paraplegic patients represents the overwhelming majority of the isolates from the neurological department

2 *Frequency of Individual Phage Types*

A survey of the material shows that some phage types were more frequent than others These were the types 8, 12 and 2, 7, 14, 16 and related types

3 *Nosocomial Occurrence*

Only a few of the strains indicate a nosocomial spreading within the hospital or ward No evidence of relatedness was found for the strains from the departments of dermatology, medicine, neurology, neurosurgery, obstetrics and gynecology, or ophtalmology The oto-rhino-laryngology department had two cases that were possibly related, one was isolated on 19 June, 1968, and had the type O 3–p29 –φ8/5 7 12* and one found 23 September, 1968 had the type O 3–p35–φ5 8,12/11 The surgical department B had more frequent occurrence of the otherwise rare serogroup 3 but the pyocine types and phage types were mostly different

Date	Sero-group	Pyocine type	Phage type
July 21 1965	3	1	7/2 3 7 8
September 25 1965	3	5	8/5 10 12
January 26 1966	3	3	12/8
February 2 1966	3	3	12/8
January 5 1967	3	35	7 8 12/5
June 15 1968	3	3	3/8

Only the infections which occurred in 1966 were clearly related

In the department of neurology, three strains isolated in November, 1965, were of the phage types φ8, φ8, and φ1,8,12 respectively This would indicate a relationship, but the serogroups and pyocine types in the same order were O 14–p19, O 9–p1, and O 6–p5

In the department of pediatrics, the phage types φ8,12, φ8, and φ12 were particularly frequent Sets of interrelated strains are shown in Table 4

The department which seemed to have the most prominent nosocomial problem was the surgical department A (Table 5), where the isolates with the serogroup O 3, pyocine type p5, and phage type φ2,7,14,16 and related patterns were most frequent This entity occurred 10 times in 1965, but even in 1966 one patient was infected with this recurrent type

During 1965–1966, in the surgical department A, there was repeated occurrence of isolates with the phage susceptibility patterns φ1,8,12/7, φ1,8,9,12/5, φ1,8,12/7, and φ1,8/7 These all belonged to the serogroup O 6 and the pyocine type p3 except for one strain with the pyocine type p5 Compared to the new set, the phage patterns obtained with the set of *Lindberg et al* on these strains were considerably longer φ119,1214,F8 Col18, φ7,31,73,Col11,Col18/21,24,F8,M6, φ7,16, 44,109,1214 F8 F10,Col121/68,73 119x F7, Col18, and φ7,119x/24,68,73,109,M6 Col18 listed in the same order as above It also appears that the results with the new typing set are more consistent, *i e* with less variation

4 *Comparison of the Two Phage Typing Sets*

In the above paragraph and in Table 5 are given the parallel results of phage typing by the new procedure of the author and the technique of *Lindberg et al* It is apparant that the phage susceptibility patterns of the

* In the text, the serogroup, pyocine type, and phage susceptibility pattern are separated by a hyphen each in the order indicated The O signifies the serogroup, p the pyocine type, and φ the phage type

TABLE 4 *Partly Interrelated Strains from the Department of Pediatrics*

Date of isolation	Clinical source	Serogroup	Pyocine type	Phage typ (new set)
June 9 1965	urine	6	3	8 12/5
October 9 1965	urine	6	3	8/7 18
October 12 1965	urine	SA*	1	12
November 1 1965	urine	9	1	12
January 29 1966	urine	6	3	10 12
February 6 1967	nose/throat	6	3	8
July 28, 1967	urine	6	3	7 8 10 12/1
April 4 1966	throat	1	50**	8/3
February 8 1966	urine	9	1	8/6
April 12, 1966	pus	1	50	8/6
October 6 1965	urine	14	33	6
October 9 1965	nose/throat	14	33	6
October 15, 1965	conjunctiva	14	8	6

* SA – Self agglutinable
** The pattern of inhibition for the indicator strains 1 to 8 in pyocine type 50 is + + +—+—+

TABLE 5 *Typing Patterns of Strains from the Surgical Department A which Seem Nosocomially Relat*

Date of isolation	Clinical source	Serogroup	Pyocine type	Phage susceptibility patte	
				New set	*Lindberg et al* set (24 2
September 1 1965	trachea	3	5	2 7 16/14	2 7 24 Col11/F7§
September 3 1965	trachea	3	5	2,7 16/14	2 7 F7 Col11
September 16 1965	trachea	3	5	2 7/8	7 21 119x Col11 Col18/?4 68 1214 F7
October 13 1965	trachea	3	5	2 7/8	31 119x Col11 Col18/7 21 24 35? M6 Col21
19 1965	trachea	3	5	7/3 8	7 21 119x Col11 Col18/24 68 F7 F10
November 10 1965	trachea	3	5	7 16/2 8	7 21 24 119x Col11 Col18/ F7 M6 Col?1
November 29 1965	trachea	3	5	2 7 14 16	7 21 119x Col11 Col18/24 68 F7,M6
December 6 1965	trachea	NT*	5	2 7/14 16	7 16 21 24 68 73 119x F10 Col11 Col18/109 M6
December 11 1965	urine	SA**	5	2 7 16/14	24 31 Col18/68 1214 F7 Col11
December 18 1965	trachea	3	5	2 7 14 16	2 7 21 119x Col11
March 1 1966	trachea	3	5	7/2 8	31 44 Col118 Col21/352
June 9 1966	surg wound	3	5	2 7/16	7 21 119x Col11 Col118/24 68 1214 F7 M6 Col121
March 17 1966	trachea	3	5	2 7 16/14	2 119x Col11/68 73 1214 1
October 11 1966	trachea	3	5	2 7 16/8 15	21/7 21 73 1214 119x Coll
December 27 1967	trachea	3	5	7/2 8 14 16	2 21 24 31 119x F7 Col11 C
January 2 1968	trachea	3	5	2 7 14 16/8 15	2 21 31 Col11 Col18

* NT = Non typable
** SA = Self agglutinable
§ The reactions with less than 20 plaques have been indicated after the oblique line

TABLE 6 *Typing Patterns of Epidemiologically Unrelated Strains from the Surgical Department A*

Clinical source	Serogroup	Pyocine type	Phage sensitivity pattern	
			New set	*Lindberg et al* set (24, 25)
Trachea	SA*	1	4,8,12,15/16	7,16 21 24,44,68,109,119x,352,1214, F8,M4,Col11,Col18/73 F7,F10, Col21
Pus	NT$	2	7	109,119x,F8 Col18/73
Pus	6,14	NT	Aux3	68,109,119x,F7,F8,F10,M4,Col11, Col18/352,1214,Col121
Pus	NT	1	Aux2	2,7,24,M4
Urine	9	29	5,7,8,12/2	2,7,16,31,44,109,352,1214 F8 Col18
Pus	NT	5	5,6,12,16/8,18	7,16,24,44,109 352,1214 F8 Col21/ 68 73,M4
Urine	NT	2	NT	7,16,14,109,F8 Col21/21,73 Col18
Expectorate	2	1	12,14,19	21,1214,Col21/31,44,119x,F7,F8
Urine	5	1	5 8,12/17	7,24,352,1214/68 F7,F10 M4
Pus	10	1	/4 14	NT
Urine	6	1	8,12,14/10	NT

* = Self agglutinable
$ = Non typable

new typing set are considerably shorter than those obtained with the set of *Lindberg et al* and that the former exhibits less variation in phage patterns in related strains It is also suggested from the results of Table 5 that the relatively high lytic activity possessed by the phages of *Lindberg et al* leads to longer phage typing patterns and as a consequence demonstrate less clear pattern differences than the new typing set

For comparison with the patterns of related strains in Table 6 are listed the results of the remainder of the strains isolated in 1965 and 1966 from the surgical department A These are all unrelated to each other, as evidenced by serology pyocine typing and the phage susceptibility pattern The findings on these strains further confirm the circumstance that the new phage typing set exhibits shorter and more distinctive patterns than the phage typing set of *Lindberg et al*

5 *Stability of Typing Patterns*

The stability of the typing patterns is of prime importance This problem may be evaluated on serial isolates from the same patients In Table 7 are listed the types of strains from patients where 4 or more isolates have been obtained It may be noted that in these patients, the cultures derive partly from relatively long periods of time Patients with few isolates obtained within shorter timespans are shown in Table 8 The data indicate that by the new phage typing set, the phage type is a relatively reproducible characteristic compared to the results with the set of Lindberg et al Related phage types have been found in some instances even when either the serological or pyocine types indicated dissimilarities

DISCUSSION

The phage types φ2,7,8,14,16, and φ8 or φ8,12 and related patterns were frequent in this material These types have also been dominating in a previous animal study (5) and among Polish hospital strains (8, 19)

At this hospital pseudomonas does not seem to be a generally recognizable nosocomial problem The few instances of associated isolates of similar types have mostly been of limited duration and extention There are two exceptions The pediatric department had a predominance of certain

TABLE 7. *Typing Patterns of Strains from Patients with Four or More Serial Isolates*

Patient	Serogroup	Pyocine type	Phage susceptibility pattern
1	NT*	5	2,7/14,16
	3	5	7,16/2,8
	3	5	2,7/14,16
	3	5	2,7/16
	3	5	2,7,16
	3	5	2,7/14,15
2	6	1	8 12
	1	39§	2,3,7,12,14,16
	3	5	7/2,8
	6	1	8/12
	6	8	8,12
3	9	3	10,12
	6	3	7,8,10,12/1
	5	1	7,8,12/2,10
	6	3	1,7,8,12/4,5,13
4	5	47§	10
	6	3	10,12
	1	3	10,12
	3	3	12/8
5	3	5	2,7,16
	3	5	2,7,8,14/16
	3	5	2,7,14,16/8,15
	3	5	2,7,14,16
	3	5	2,7,14,16/8,15
6	6	3	8,10,12
	6,14	3	8,10 12
	6	3	10
	6	3	8,12
7	6	3	1,7,8,12
	6	29	5,7,8,12
	6	3	1,7,8,12
	4	15	5,7,8,10,12
	13	29	5,7 8,12
	6	40§	7,8,12
	SA**	3	1,7,8,12
	6	7	1,7,8,12
	6	5	1,2,6,7,8,12,16
	6,14	5	1,6,7,8,12
8	8	10	4,15
	8	10	/15
	8	10	Aux3
	8	10	Aux3
	14	8	6
	14	8	6
	NT	1	/8
9	NT	43§	/8
	NT	1	7,8/4
	14	17	8
	9	1	8

* NT = [illegible]pable ** SA – Self agglutinable
§ The inhi[illegible] patterns of some pyocine types indicated were not given individual numbers by *Gillies & Govan* ([illegible]
The inhibitio[illegible]n of p39 was – + + + – – + +, p40 was + + – – + – – –,
p43 was – [illegible] + – + +, p47 was + + + + + – + –

TABLE 8 *Typing Patterns of Strains from Some Patients with Three Serial Isolates*

Patient	Serogroup	Pyocine type	Phage susceptibility pattern
A	6	5	1,8,12/7
	NT*	5	5,6,12,16/8,18
	9	29	5,7,8,12
B	6,14	NT	Aux3/12
	NT	2	/7
	NT	2	/7
C	3	5	2,7,8
	3	5	7/3,8
	3	5	2,7,14,16
D	5	1	10/3
	3	1	8,12
	1	52$	4,5,8,12/7
E	10	22	10
	6	3	1/8
	6	3	2,8,9,10,12,14,15
F	9	38$	14,15/2
	3	9	2,4,9 10,12,14,15,16/3,7,8
	3	9	2,4,9,10,12,14,16/3,8,11,15
G	6	3	3,8,12
	14	8	6
	6	3	3,8,12
H	6	5	8
	6	27	8
	5	58$	6,8
I	6	3	8
	2	3	8
	6	3	8
J	6	22	1,8
	6	3	1,8
	6	3	1,8
K	3	1	/18
	NT	1	/18 14,15
	NT	1	/18
L	6	3	1,7,8
	6	3	1 8
	3	22	1,7,8

* NT = Non typable

$ The pyocine types correspond to the following inhibition patterns p38 – + + – – – + –, p52 – – + + + – – –, p58 – – + – + – –

types particularly among urinary tract infections Considerably more serious was the situation in the surgical department A where the type 0 3-p5-φ2,7,8 14,16 was found in specimens from the trachea during the period from September, 1965 to January, 1968, i e during the entire duration of this investigation The fact that these patients were mostly tracheotomized points to an infectious reservoir in the environment possibly in the respirators

A frequent finding of pseudomonas in tracheal secretion has been noted previously by *Sutter et al* (38, 39) in patients with tracheostomy

One very interesting feature of the present data is that the new pseudomonas bacteriophage typing set developed by the author (2, 3) compared rather favourably with that of the system of *Lindberg et al* (24, 25) (Tables 5 and 6) The new set exhibited a pattern stability which was better than that

shown by the set of *Lindberg et al* It had shorter pattern codes than the latter system These observations were confirmed in a Polish hospital study where the set of *Lindberg et al* was handled by another worker (*19*), but the new phage typing set by the present author The set of *Lindberg et al* also rendered long pattern codes in the hands of others as is apparent from a nosocomial study by *Lowbury et al* (*26*) In using the *Lindberg et al* set *Lowbury et al* found it convenient to differentiate between constant reactions and phage susceptibilities which were variable within groups of related bacterial strains In a study from New Zealand, *Knights et al* (20) found short pattern codes with the set of *Lindberg et al Lindberg et al* themselves to a certain extent recognized long phage type codes (25)

The shorter codes of the new phage typing alternative is of practical importance since it thereby contributes to a more rationalized recording of results In addition, pattern codes as long as shown repeatedly by the set of *Lindberg et al* in this work, make strain relatedness more uncertain, when two strains susceptible to a large number of phages share the majority of reactions and differ in a few as is the case *e g* in some instances of Table 6 it is difficult based on phage types alone to conclude that the two strains are entirely different

The finding that the new phage typing set successfully types more bacterial strains than the set of *Lindberg et al* also justifies a preference for the new set In the Polish hospital study out of the 42 strains documented in the Tables (19) 13 were non typable by the set developed by *Lindberg et al* but typable by the new set whereas only 3 isolates were non typable by the new set and still typable by the set of *Lindberg et al* In 7 instances strains were non typable by both systems

The usefulness of the three typing procedures in an epidemiological context has been evaluated in epidemiologically related strains collected at a restricted area over a short time span 8 1 the present results on epidemiologically less related strains also allow interesting conclusions

A suitable typing procedure must render stable types It is the impression that the serogroup is a stable character The serogroup differs from the expected (as evaluated by similarity in the two other typing systems) only in a few instances (*e g* patients J and L in the Table 8) Erroneous observations may be the cause but serological variability may also be indicated The fact that such variability may occur in serial isolates from the same patient is shown for instance in the patient no 9 in Table 7 or no H in Table 8 These show what appears to be a gradual transition with a stepwise change in each of the typing systems individually which eventually leads to a new composite pattern

The pyocine type showed more variability than the other typing procedures in instances when these indicated that the strains were related Nevertheless the pyocine typing method of *Gillies & Govan* has been found to have a reasonable reproducibility for the individual strain (15), and is thus preferable to other alternatives which work with different cultivating conditions (41)

Besides type stability, another important aspect is typability In Table 1 are shown the percentages of strains typed with each of the three classes of typing procedures as obtained by different investigators Pyocine typing and phage typing generally have showed similar typabilities, serogrouping has mostly typed more strains It is notable that the new phage typing set had better typability than any of the other sets listed, an observation which was also made when several sets previously were applied simultaneously to the same bacterial strains (3) The *Gillies & Govan* pyocine typing procedure in my hands typed more strains than has been reported by others Also on animal pseudomonas strains the *Gillies & Govan* set has been applied with good results (43) typing 89 6 per cent

Serogrouping and pyocine typing alone however have not been sufficiently discriminatory since they only subdivide the majority of strains in a few types In the

Habs O grouping system three to four groups represent 60–80 per cent of the bacterial population Similar results have been obtained with other grouping schemas The pyocine typing methods subdivide the pseudomonas strains in 3 major groups totalling some 75 per cent In animals p1 has accounted for 66 per cent of the strains (43)

Phage typing alone on the other hand renders more subgroups When the patterns are interpreted with the safety margin by which maximally 2 reactions may be different (4), phage typing with the new typing set represents a relatively reliable typing method for serial cultures The reproducibility of the phage typing method has been elaborated previously (4) Although phage typing has its limitations compared to the alternative methods as has been done presently it is a better alternative

An interesting comparison would be the new phage typing set and the serotyping system of *Lanyi* (22 23) The latter in the hands of the originator leaves a mere 0 7 per cent untypable if only O grouping is performed The lack of subdivision into smaller entities by the Habs and other serogrouping systems is ameliorated in the Lanyi system by a slightly more improved subdivision of the O antigens and by employment of a system of 7 H antigens in addition Thereby *Lanyi* has succeeded in subdividing 497 strains in as much as 53 serotypes The technical difficulties involved in H typing of pseudomonas is not to be underestimated but considering the elaborate nature of phage typing the *Lanyi* serotyping scheme seems to represent an *interesting alternative* Another immunological schema based on a combination of O and H antigens is the *Verder Evans* system (40) It is difficult to obtain an adequate basis for a comparative evaluation of the two systems from available literature alone but the Lanyi system has been shown to render adequate subdivision and is reasonably reproducible

One important feature of the present findings is that the types obtained by each procedure could be further subdivided by the other methods Consequently the joint use of two typing procedures would be useful in the epidemiological typing of pseudomonas The question is what methods may advantageously be combined

Csiszar & *Lanyi* (12) considered the combination of serological procedures and pyocine typing to render a reliable differentiation *Wahba* (41) and *Bassett et al* (1) likewise employed a combination of serogrouping and pyocine typing A previous communication of the author concluded (6) that better results would be obtained with a combination of serology and pyocine typing than evolved with each method alone Phage typing can more readily be used alone but the precision of the results will improve when supplemented by another procedure preferably serogrouping *Gould* & *McLeod* (16) and *Jedlickova and Pillich* (18) both without reservation claimed that serogroup specificity and phage susceptibility were related but their results were obtained in a small number of bacteria that in addition were epidemiologically related and consequently did not justify such generalisations A previous report based on a larger number of more unrelated strains (7) showed little tendency for strains lysed by certain phages to belong predominantly to one or two serogroups

In summing up the experiments with three typing procedures for *P aeruginosa* it would seem that phage typing with the new set is the most suitable single epidemiological typing procedure Phage typing however will benefit from a combination with serogrouping Phage typing is only recommended for the larger laboratories with sufficient numbers of pseudomonas strains to make feasible the effort of maintenance and the more complicated typing procedure as such When only a few strains of pseudomonas need be typed per week immunological characterization would seem the method of choice Pyocine typing is simple to handle, suitable even for modest facilities and reasonably reproducible in the modification of *Gillies* & *Govan* (15) A combination of serogrouping and pyocine typing probably is comparable in differentiation to phage typing

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ANTAGONISTIC ACTION OF ALPHA HAEMOLYTIC STREPTOCOCCI ON *NEISSERIA ELONGATA*

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Alpha haemolytic streptococci have a marked inhibitory effect on *N elongata* in blood agar cultures *M nonliquefaciens* and *M osloensis* appear to be less affected and *M phenylpyruvica* and *N catarrhalis* are quite unaffected The inhibition only appears on the crowded areas of the cultures and not when colonies grow separately It appears to be a question of competition for growing space rather than a toxic effect The possible consequences of such antagonism for the detection of certain species in cultures are discussed

This study was inspired by an accidental observation in the course of attempts to isolate *Neisseria elongata* (5) from pharyngeal cultures From a primary culture several Neisseria like colonies were picked and subcultured in sectors on a new blood agar plate One of these sectors gave growth of small gram negative oxidase positive rods later identified as *N elongata* in mixture with alpha streptococci The strain was designated 516/72 (2) Material from this sector was spread on a new plate in order to obtain growth of isolated colonies and this second subculture presented a rather striking phenomenon In the area of dense growth, practically only a carpet of alpha streptococci could be seen with occasional small patches where growth of *N elongata* could be detected The culture gave the impression of a practically pure culture of the streptococci On the area where the organisms grew with separate colonies there was growth of about equal numbers of colonies of *N elongata* and streptococci (Fig 1) The phenomenon suggested an antagonistic effect of the streptococci against the Neisseria In order to verify this some experiments were carried out

EXPERIMENTS AND RESULTS

Exp 1 Approximately equal quantities of strain 516/72 and the streptococcus were picked up with the same loop and the mixture was spread on a blood agar plate On the next day a patchy but very marked inhibition of strain 516/72 was seen in the crowded area of the plate whereas colonies of both organisms grew side by side where the colonies had become separated

Exp 2 Strain 516/72 was streaked in closely spaced parallel streaks on a blood agar plate, and the streptococcus was streaked in one streak at right angles across the Neisseria streaks On the next day the streptococcus streak showed apparently pure growth of streptococci with complete inhibition of

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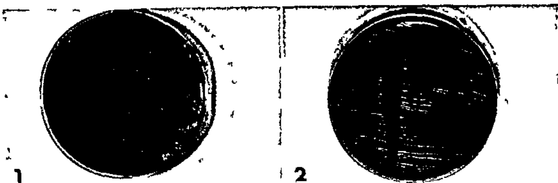


Fig 1 Secondary culture from mixed culture of *Neisseria elongata* and alpha haemolytic streptococci In the crowded area practically only streptococci can be seen Where colonies are well separated colonies of *N elongata* (oxidase positive) are numerous

Fig 2 *N elongata* has been inoculated in parallel streaks across a blood agar plate On the left side where a drop of streptococcus culture has run down the plate growth of *N elongata* has been nearly completely inhibited On the right side a drop of sterile broth which has run down the plate has left no trace

strain 516/72 which however grew well right up to the margin of the streptococcus streak The experiment was repeated with a mucoid pneumococcus instead of the streptococcus and again the growth of strain 516/72 appeared to be completely inhibited corresponding to the pneumococcus streak Microscopically this area appeared to contain only pneumococci and gram negative rods were not seen Repetition of the experiment with a total of 17 different viridans streptococci all isolated from pharyngeal cultures gave the same result complete or nearly complete inhibition of strain 516/72

Exp 3 It seemed possible although unlikely that enough of the Neisseria inoculum could have been removed in the act of cross streaking with the streptococcus to give a false impression of growth inhibition The technique was accordingly modified thus a plate was inoculated with parallel streaks of the strain 516/72 The plate was placed in an inclined position with all the streaks level and a drop of an overnight culture of the streptococcus in Todd Hewitt broth was allowed to run down one side of the plate at right angles to the streaks A drop of sterile medium was similarly allowed to run down on the other side of the plate The next day there was complete inhibition of strain 516/72 where the drop of the streptococcus culture had run down whereas the drop of uninoculated medium had left no trace at all (Fig 2)

The experiment was repeated with 10 fold dilutions of an overnight streptococcus culture Complete inhibition of strain 516/72 was obtained with undiluted culture and with dilutions 1 10 and 1 100 but higher dilutions produced no inhibition

Exp 4 In order to study whether alpha streptococci would also inhibit the growth of other members of the *Neisseriaceae*, the same technique as in the preceding experiment was used with different strains of *N elongata Moraxella* and *N catarrhalis* Many of these experiments were repeated one or more times with slightly diverging results but with clear cut trends

Seven strains of *N elongata* 1488/72 I II and III, 1737/72 I II and III and 1502/72 (2) gave similar results as strain 561/72 complete or nearly complete inhibition by alpha streptococci

Of 7 strains of *Moraxella* isolated from the nose, and presumably *M nonliquefaciens* (1), 6 were partly to nearly completely inhibited In three of these cases subcultures from the inhibited area gave growth only of streptococci and in two cases there was a great

predominance of streptococci in the subcultures The type strain, 4663/62 (3) was apparently completely inhibited and a microscopic film from the inhibition zone revealed practically only streptococci with a few scattered, more or less bizarre gram-negative cells

The type strain of *M osloensis*, A 1920 (3) was apparently only partly inhibited in a patchy manner

The type strain of *M phenylpyrouvica*, strain 2863 (4) showed no inhibition, and 5 strains of *N catarrhalis*, isolated from nose cultures, showed no inhibition or even, in one case, apparent growth improvement

DISCUSSION

Antagonism between microorganisms probably is very common and may be thought to be a very important factor in deciding the microflora in various ecosystems, including the mucous membranes of man, but little factual knowledge appears to be available about this Thus very little appears to be known about why certain bacterial species are more or less restricted to certain, often very limited localities The bacterial flora of the human respiratory tract seems to be a good illustration of this Thus studies have shown that *M nonliquefaciens* and *N catarrhalis* are very common in the nasal cavities (1), but comparatively rarely found in the pharynx Alpha streptococci and various *Neisseria* species, and *Micrococcus mucilaginosus*, on the other hand are extremely common in pharyngeal cultures, but are only very rarely isolated from the nasal cavity, only a few centimeters away Many causal factors probably are behind such phenomena and antagonism between species may be one such factor, or set of factors

Whether the antagonism studied in this paper is a pure *in vitro* phenomenon or also may have any relevance to the considerations just mentioned is quite unknown In any case such phenomena might have a bearing upon bacteriological diagnosis It is conceivable that a small number of organisms such as *N elongata*, and possibly *M nonliquefaciens* would be more difficult to detect in the presence of a large, than of a small number of alpha streptococci One might even suggest the possibility that such antagonism might create false impressions as to the frequency of such organisms in the pharynx

The results indicate that some organisms may be highly susceptible to such antagonisms, others less susceptible and others again not at all The mechanism of the described phenomena of inhibition is unknown It does not appear to be a question of a diffusible principle, since the inhibited strains grow right up to the inhibiting strain, but perhaps rather of a competition for growing space, where certain organisms, in this case alpha streptococci, for some unknown reason have the advantage, possibly by creating unsuitable growth conditions for the competitor

It might be rewarding to pursue these studies further and to include other species in the material, but the intention of this paper is only to call attention to the phenomenon Similar observations may have been made before, but if so, such reports have escaped our notice

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SEROLOGICAL CROSS-REACTIONS BETWEEN DIFFERENT *BRUCELLA* SPECIES AND *YERSINIA ENTEROCOLITICA*

Biological and Chemical Investigations of Lipopolysaccharides from Brucella abortus *and* Yersinia enterocolitica *type IX*

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Hot phenol water extraction of *Brucella abortus* (B a) and *Yersinia enterocolitica* type IX (Y e) cells was performed The results from the chemical analyses of fractions 3 (F3) and 5 (F5), which were recovered from the aqueous and the phenol phase respectively, show that these fractions contain lipopolysaccharide complexes A clear-cut difference was noted in the yield expressed as the obtained dry weights from fractions F3 and F5 for B a and Y e For B a the yield was greater in F5 than in F3 The relation was about 3 1 For Y e the reverse was the case, the relation being about 1 14 The F5 fractions from both B a and Y e were highly immunogenic They induced leucopenia and leucocytosis if injected into rabbits This indicates that both fractions have an endotoxin effect Both fractions contained O somatic agglutinogen which if injected into rabbits, gave rise to both primary and secondary immune responses The antibodies produced agglutinated both B a. and Y e bacteria In contrast neither the F3 fraction from B a nor that from Y e induced leucocytosis or gave rise to agglutinins in rabbits

Recently, *Ahtonen et al* (1969) demonstrated a strong serological cross reaction between *Brucella abortus* and *Yersinia enterocolitica* type IX Later investigations indicate that the antigenic determinants common to *Brucella* and *Y enterocolitica* type IX seemed to be present in the lipopolysaccharides of respective bacteria (*Diaz et al* 1970, *Hurvell* 1972)

In the present investigation, extractions of whole bacteria from *B abortus* and *Y enterocolitica* type IX with the hot phenol water method were employed in order to obtain fractions containing lipopolysaccharides and to compare these fractions of the two species from different points of view

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MATERIALS AND METHODS

Bacterial Strains

B abortus strain 544 (B a) and *Y enterocolitica* type IX strain MY 79 (Y e) were employed and have been used in a previous study (*Hurvell* 1972) Cultivation and harvesting were performed as described earlier (*Hurvell* 1972)

The cells were killed by adding formalin to a final concentration of 1 per cent and stored over night at 4° C The cells were then washed three *times with cold 0 15 M NaCl and lyophilized*

Extraction Method

By extraction with hot phenol water, fractions from the aqueous and the phenol phase were prepared from whole bacteria of B a. and Y e, as

described in detail by *Redfearn* (1960), *Baker & Wilson* (1965 b), and *Leong et al* (1970) The principal features of the extraction procedure are shown in Fig 1 The designations of the fractions are those used by *Redfearn* (1960) and *Leong et al* (1970)

Agglutination Test

The preparation of the antigens and the performance of the test have been described in detail earlier (*Hurvell et al* 1971) Serial two fold dilutions of serum were prepared in 0 15 M NaCl. Heat killed whole cells were used as antigens The incubation was performed overnight in a water bath at 52° C The agglutination was read optically

Primary and Secondary Antibody Response

The immunogenicity of fractions F3 and F5 from B a and Y e obtained by the hot phenol water extraction was tested as follows From each fraction 12 5 µg were dissolved in 1 ml of pyrogen free 0 15 M NaCl (ACO Lakemedel, Stockholm, Sweden) and injected intravenously in rabbits Four rabbits per fraction were used Ten days later the animals were bled (primary bleeding) The same day a booster dose of 12 5 µg was administered Twelve days after the booster injection the rabbits were bled again (secondary bleeding)

Leucopenic and Leucocytotic Test

To test the effect of fractions F3 and F5 from B a and Y e on the number of circulating leucocytes rabbits weighing on an average 3½ kg were used From B a an amount of 100 µg from each fraction in 1 ml of pyrogen free 0 15 M NaCl (ACO Lakemedel AB Stockholm, Sweden) was injected From Y e the corresponding dose was 5 µg The employed amounts of 100 µg and 5 µg are the most suitable doses of the respective fraction F5 which could be injected without producing mortal toxin reactions in the rabbits Three rabbits per fraction were used About 1 ml blood from the ear vein of the rabbits was taken before and at several intervals after injection of the test materials The blood samples were stabilized with formalin and Titriplex III ® (Merck, Darmstadt, W Germany) by the method of *Tolle & Jahnke* (1965) After haemolysis with Cetrimide® (Fluka A/G St Gallen Switzerland) as described by *Peterson & Karlen* (1963) the leucocyte count was determined with an electronic particle counter (Celloscope 101, AB Lars Ljungberg & Co Stockholm Sweden) Duplicate counts of each sample were performed and averaged The total error of the method was considered to be less than (±) 3 5 per cent under the experimental conditions (*Thorell* 1963) The percentage of white blood cells (WBC) was calculated from $t_x/t_0 \times 100$ = per cent WBC (*Leong et al 1970*), where t_x was the leucocyte count at a given interval after and t_0 the leucocyte count before administration of the test dose The WBC count before injection of the fractions was taken as 100 per cent Values lower than 100 per cent represented leucopenia and values higher than 100 per cent represented leucocytosis

10 µg of commercially available lipopolysaccharides from phenol water extractions of *Escherichia coli* 055 B5 as well as of *Salmonella typhimurium* (Difco Laboratories, Detroit USA) were dissolved in 1 ml of pyrogen free 0 15 M NaCl and used as positive controls Rabbits injected with 1 ml of the pyrogen free NaCl were used as negative controls

Chemical Analyses

Carbohydrate The carbohydrate content of fractions F3 and F5 was determined by the phenol sulphuric acid procedure described by *Nowotny* (1969)

2 keto 3 deoxyoctulosonic acid (KDO) The KDO values were determined by the semicarbazid technique of *McGee et al* (1954) with the ammonium salt of KDO as the standard of reference An amount of 200 µl of the samples dissolved in H_2O was mixed with 200 µl reagent (200 mg semicarbazide/HCl, 300 mg sodium acetate, 3 H_2O 20 ml H_2O) and kept at 30° C After 15 minutes 1000 µl H_2O were added and the absorbance was measured at 252 and 260 nm

Nitrogen The quantitative determinations of nitrogen were performed as described by *Lowry et al* (1951)

Phosphorus The phosphorus contents were determined colorimetrically by the method of *Allen* (1940) The amount of P present is read from a calibration curve obtained with standard solutions of KH_2PO_4

Ultraviolet absorption For the detection of ultraviolet (UV) absorbing substances in fractions F3 and F5 from the phenol water extractions a scanning spectrophotometer (Beckmann Dk 2) was used The specific absorption at 260 nm was determined and the contaminating ribonucleic acid (RNA) was quantified with a purified preparation of RNA (Worthington Biochemical Corp Free hold New Yersey USA) as a standard of reference Fractions which contained more than 10 per cent (w/w) of RNA were treated with ribonuclease (Ribonuclease 5×Crystalline, Nutritional Biochemical Corp, Cleveland Ohio USA) The fractions were dissolved to a concentration of 10 mg/ml (w/v) in 0 1 M acetate buffer pH 5 0 Ribonuclease was added to a final concentration of 20 µg/ml The mixture was dialysed (Seamless cellulose tubing Union Carbide Corporation Chicago, USA) against 0 1 M acetate buffer with pH

5 0 at 25° C under cont nous stirr ng for 24 hours New scann ng with the Beckmann Dk 2 was per formed

RESULTS

Fig 1 gives a flow sheet of the used extraction method The yield and the dry weights of the respective fractions will be seen from Table 1 For both *B abortus* (B a) and *Y enterocolitica* type IX (Y e) the main part of the yield was obtained in fractions F1 and F2 F1 represents the interphase layer between the aqueous phase and the phenol phase and F2 is the precipitate of bacterial cell residue after the fractionation (see Fig 1) F1 plus F2 comprised for B a 55 0 per cent and for Y e 67 1 per cent of the original material Fractions F3 and F4 were obtained from the aqueous phase Here F3 predominated both for B a and for Y e the yield being 1 5 per cent and 4 9 per cent respectively The rest of the fractions were obtained from the phenol phase There was a marked difference in the yield of fraction F5 from the phenol phase the value being 4 4 per cent for B a and 0 4 per cent for Y e

The contents of RNA were lower than 0 5 per cent (w/w) for both F5 fractions RNA peaks were obtained at the UV scan

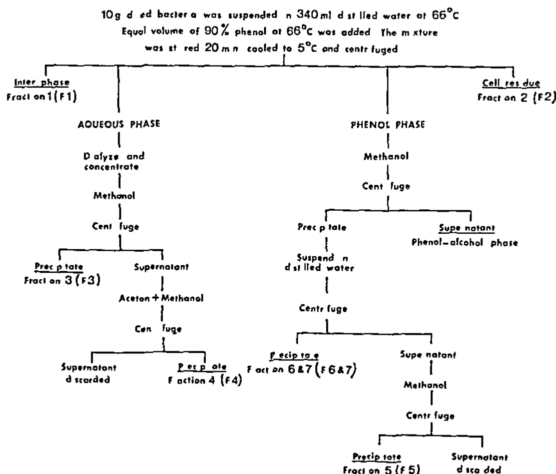


Fig 1 Fract onat on steps follow ng extract on of *B abortus* and *Y enterocol tica* type IX by the hot phenol-water method

TABLE 1 *Yields and Dry Weights of the Different Fractions Obtained from* B abortus *and* Y entero-*colitica type IX by the Hot Phenol Water Extraction*

Fractions	*B abortus*		*Y enterocolitica*	
	g dry weight	per cent	g dry weight	per cent
1	3 6616	34 46	0 5740	5 56
2	2 1845	20 56	6 3753	61 75
3	0 1630	1 53	0 5028	4 87
4	0 0234	0 22	0 0125	0 12
5	0 4668	4 39	0 0363	0 35
6 & 7	1 1411	10 74	1 3433	13 01
Phenol alcohol phase	0 7171	6 75	0 5776	5 59
Total yield	8 3575	78 65	9 4218	91 25
Starting material	10 6255		10 3248	

TABLE 2 *Chemical Composition of the Different Fractions Obtained from* B abortus *(B a) and Y enterocolitica type IX (Y e) by the Hot Phenol Water Extraction*

Fractions	Nitrogen per cent	Phosphorus per cent	Carbohydrate per cent	KDO* per cent
B a 1	15 5	1 4	5 8	–†
2	14 2	1 3	2 8	–
3§	2 5	1 0	34 8	6 5
4	2 1	1 5	51 3	–
5	3 4	0 5	19 6	8 4
6 & 7	7 3	0 1	0 4	–
Phenol alcohol phase	5 0	2 5	2 3	–
Y e 1	5 7	1 6	4 5	–
2	6 1	1 1	2 1	–
3§	0 7	1 2	24 5	2 2
4	2 2	6 5	12 8	–
5	2 5	0 5	22 8	4 3
6 & 7	7 3	0 2	1 6	–
Phenol alcohol phase	5 1	1 6	0 8	–

* KDO 2 keto 3 deoxyoctulosonic acid
§ The analyses are performed after treatment with ribonuclease
† Not performed

ning of fraction B a F3 and Y e F3 Their contents of RNA were 75 per cent and 31 per cent After treatment with ribonuclease, the values fell to 8 per cent and 5 per cent, respectively *

The results from the quantitative chemical analyses are recorded in Table 2 In comparison with the other fractions, fractions 3, 4 and 5 have a noticeable predominance of carbohydrates The results of the analyses of the KDO content in fractions F3 and F5 varied between 2 2 per cent and 8 4 per cent The two fractions from B a had the highest content

The results from the immunization tests with fractions F3 and F5 from B a and Y e,

* All the tests and analyses with the F3 fractions reported in the following sections were made after the ribonuclease treatment

TABLE 3 *Agglutinin Titres of Antisera from Rabbits Immunized with Fraction F3 and F5 of* B abortus *(B a) and* Y enterocolitica *type IX (Y e)*

Fractions	Agglutinin titres			
	Primary response		Secondary response	
	B a antigen	Y e antigen	B a antigen	Y e antigen
B a F3	< 20	< 20	< 20	< 20
B a F5	640	640	1 280	1 280
Y e F3	< 20	< 20	< 20	< 20
Y e F5	160	320	1 280	5 120

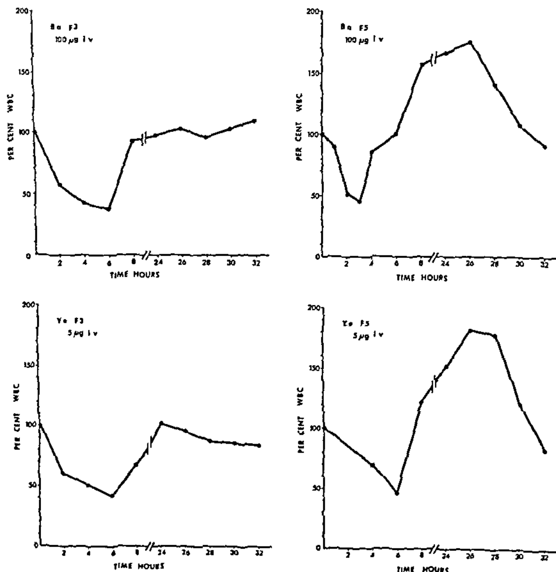


Fig 2 Leucopenic and leucocytotic effects of *B abortus* (B a) and *Y enterocolitica* type IX (Y e) fractions 3 (F3) and 5 (F5) in rabbits.

TABLE 1 *Yields and Dry Weights of the Different Fractions Obtained from* B abortus *and* Y entero-*colitica type IX by the Hot Phenol-Water Extraction*

Fractions	*B abortus* g dry weight	per cent	*Y enterocolitica* g dry weight	per cent
1	3 6616	34 46	0 5740	5 56
2	2 1845	20 56	6 3753	61 75
3	0 1630	1 53	0 5028	4 87
4	0 0234	0 22	0 0125	0 12
5	0 4668	4 39	0 0363	0 35
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Phenol alcohol phase	0 7171	6 75	0 5776	5 59
Total yield	8 3575	78 65	9 4218	91 25
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Fractions	Nitrogen per cent	Phosphorus per cent	Carbohydrate per cent	KDO* per cent
B a 1	15 5	1 4	5 8	–†
2	14 2	1 3	2 8	–
3§	2 5	1 0	34 8	6 5
4	2 1	1 5	51 3	–
5	3 4	0 5	19 6	8 4
6 & 7	7 3	0 1	0 4	–
Phenol alcohol phase	5 0	2 5	2 3	–
Y e 1	5 7	1 6	4 5	–
2	6 1	1 1	2 1	–
3§	0 7	1 2	24 5	2 2
4	2 2	6 5	12 8	–
5	2 5	0 5	22 8	4 3
6 & 7	7 3	0 2	1 6	–
Phenol alcohol phase	5 1	1 6	0 8	–

* KDO 2 keto 3 deoxyoctulosonic acid
§ The analyses are performed after treatment with ribonuclease
† Not performed

ning of fraction B a F3 and Y e F3 Their contents of RNA were 75 per cent and 31 per cent After treatment with ribonuclease, the values fell to 8 per cent and 5 per cent, respectively *

The results from the quantitative chemical analyses are recorded in Table 2 In comparison with the other fractions, fractions 3, 4, and 5 have a noticeable predominance of carbohydrates The results of the analyses of the KDO content in fractions F3 and F5 varied between 2 2 per cent and 8 4 per cent The two fractions from B a had the highest content

The results from the immunization tests with fractions F3 and F5 from B a and Y e,

* All the tests and analyses with the F3 fractions reported in the following sections were made after the ribonuclease treatment

would appear preferentially in the phenol phase

The results from the chemical analyses show that fractions F3 and F5 both contain LPS complexes For *B abortus* these results agree with those from earlier studies by *Redfearn (*1960) and *Leong et al* (1970) *Diaz et al* (1970) using the hot phenol water extraction obtained a proteinlipopolysaccharide KDO complex in fraction 5 from *Y enterocolitica* type IX

At the biological endotoxin test on fractions F3 and F5 from B a and Y e in the present study, different effects on the leucocyte count were obtained The respective F3 fractions lacked the ability to provoke leucocytosis in the rabbits A leucopenia followed by leucocytosis is to be regarded as a typical endotoxin effect (*Milner et al* 1971), whereas leucopenia alone is not considered to be a specific manifestation of endotoxin (*Thomas* 1954)

The results from the present studies show that an endotoxin effect was obtained in the F5 fractions both for B a and for Y e, whereas the respective F3 fractions lacked or had a nonmeasurable endotoxin effect For *B abortus* this agrees well with similar studies by *Baker* & *Wilson* (1965 a), who used changes in the serum iron concentration in mice as a parameter for the endotoxin effect *Redfearn* (1960) used the Shwartzman reaction in guinea pigs to test the toxicity of fractions 3 and 5 from *B abortus melitensis*, and *suis* He obtained marked reactions with the respective fractions 5 but only minimal dermal toxicity responses with fractions 3 According to *Redfearn*, this biological difference would be due to molecular configurations of the highly labile phenol insoluble lipid of fraction 3 and the phenol-soluble lipid of fraction 5

The results from the present immunization tests indicate that with the injection doses and inoculation intervals used, no measurable agglutination titres were obtained by injection of the F3 fractions from B a and Y e For B.a F3 these results agree with those obtained in earlier studies (*Leong et al* 1970)

The available literature contains no similar studies concerning fraction 3 of *Y enterocolitica* type IX *Davies* (1958), however, found that hot phenol-water extraction of *Y pseudotuberculosis* cells yielded lipopolysaccharides which were recovered from the aqueous phase and were poorly antigenic The antisera against the lipopolysaccharides failed to precipitate the homologous materials in fluid media and gave no line of precipitation if tested by the agar diffusion precipitin technique On the other hand *Crumpton et al* (1958) found antigenic response detected by the more sensitive haemagglutination technique with red cells sensitized with the corresponding polysaccharides

Both at the primary and at the secondary immune response after injection of fraction F5 from B a as well as from Y e, we obtained agglutinins which reacted strongly with agglutination antigen both from B a and from Y e For B a F5 the same titres were obtained against homologous and heterologous antigens For Y e F5, on the other hand, there were differences between the homologous and the heterologous titres These results are highly consistent with those obtained in previous immunization tests on rabbits, in which heat killed whole cells were used as antigen (*Hurvell et al* 1971) Accordingly, fraction 5 from both B.a and Y e contains O somatic agglutinogens, whose antigenic determinants give rise to markedly cross reacting agglutinins

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SEROLOGICAL CROSS-REACTIONS BETWEEN DIFFERENT *BRUCELLA* SPECIES AND *YERSINIA ENTEROCOLITICA*

Immunochemical Studies on Phenol-Water Extracted Lipopolysaccharides from Brucella abortus *and* Yersinia enterocolitica *type IX*

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The cross reactivity of lipopolysaccharides (LPS) in *Brucella abortus* and *Yersinia enterocolitica* type IX recovered from the aqueous and phenol phases of the hot phenol water method was examined in immunodiffusion tests The antigens and the cross reactivity were unaffected by heating and by treatment with lipolytic and proteolytic enzymes, but periodate oxidation was found to destroy the precipitating ability This indicates the carbohydrate nature of the cross reacting antigenic determinants The monosaccharide composition of the fractions was estimated using gas liquid chromatography of peracetylated alditol acetates The LPS of *B abortus* and *Y enterocolitica* type IX had glucose and galactose as common monosaccharide constituents In addition, the LPS of *B abortus* contained mannose and that of *Y enterocolitica* type IX heptose The possibility of having the cross reactivity localized in O specific side-chains is discussed

The hot phenol-water method devised by *Westphal et al* (1952) for the extraction of whole bacteria, results in the recovery of lipopolysaccharides in the aqueous phase Later investigations have shown, however, that sometimes lipopolysaccharides can also be found in the phenol phase (*Redfearn* 1960, *Baker & Wilson* 1965 b *Leong et al* 1970 *Diaz et al* 1970, *Luderitz et al* 1971) Furthermore, the lipopolysaccharide fractions isolated from the aqueous and phenol phase, respectively were found to differ in their biological activity (*Redfearn* 1960, *Baker & Wilson* 1965 a *Leong et al* 1970)

In an earlier investigation of the serological cross-reaction between *Brucella abortus* and *Yersinia enterocolitica* type IX, extraction of whole bacteria by the hot phenol water method was found to yield cross reactive material (*Hurvell* 1973) A striking difference was noted, however The phenol fractions elicited an antibody response and gave endotoxin reactions with rabbits, whereas the aqueous fractions gave no endotoxin reactions and no measurable agglutination titres were obtained under the experimental conditions

The object of the present paper was to study the immunochemical basis for the serological cross-reactivity This was done by (1) immunodiffusion with the aqueous and phenol fractions as antigens and antisera prepared by immunizing rabbits with acetone-killed whole bacteria of *B abortus* and *Y*

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enterocolitica type IX, (ii) periodate oxidation of the fractions and treatment with proteolytic and lipolytic enzymes before immunodiffusion assays, and (iii) qualitative and quantitative determination of the monosaccharide composition of the fractions by means of gas liquid chromatography of peracetylated alditol acetates

MATERIALS AND METHODS

Preparation of Lipopolysaccharides

The hot phenol water extraction and the characteristics of *B abortus* (B a) and *Y enterocolitica* type IX (Y e) lipopolysaccharides (LPS) recovered from the aqueous phase (F3) and the phenol phase (F5) have been described earlier (*Hurvell* 1973)

Antisera

The rabbit antisera to whole acetone killed bacteria of B a and Y e were the same as those employed in earlier studies (*Hurvell* 1972)

Immunodiffusion Test

The method was performed as previously described (*Hurvell* 1972) The LPS of fractions F3 and F5 dissolved in phosphate buffered 0 15 M NaCl, pH 7 2, were used as antigens The diffusion was performed at 37° C for 52 hours in a moist chamber

Heat Stability Test

The LPS from F3 and F5 of B a and Y e were suspended (5 mg/ml) in phosphate buffered 0 15 M NaCl pH 7 2, and heated for 20 minutes at 100° C in sealed glass tubes

Treatment with Enzymes

The LPS fractions F3 and F5 were treated with lipases from wheat germ (Nutritional Biochemicals Corp , Cleveland Ohio) and hog pancreas (Sigma Chemical Comp St Louis Missouri) The enzymes to substrate ratio was 1 2 in 0 15 M phosphate buffered NaCl pH 7 0 The mixtures were incubated for 18 hours at 37° C in a water bath Treatment of the LPS fractions with protease from *Streptomyces griseus* (Sigma Chemical Comp , St Louis, Missouri) was performed in 0 15 M phosphate buffered NaCl pH 7 4 for 18 hours at 37° C

The enzymes in the LPS-enzyme mixtures were inactivated at 100° C for 5 minutes The mixtures were dialysed for 2 days at 4° C against repeated changes of distilled water The preparations were lyophilized before use in immunodiffusion tests

Controls without the enzymes were handled as above and included in the immunodiffusion tests

Oxidation with Periodate

The LPS from fractions F3 and F5, respectively were dissolved in distilled water to 0 2 per cent w/v An equal volume of 0 1 M sodium periodate was added to each preparation The mixtures were incubated in the dark for 72 hours at 20° C and then dialysed against distilled water overnight at 4° C The periodate treated LPS preparations were lyophilized before use in immunodiffusion tests.

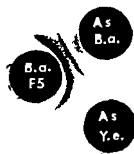
Gas Chromatographic Analysis

The analysis of the carbohydrate composition of the LPS fractions F3 and F5 from B a and Y e was based on the method described by *Sawardeker et al* (1965) The fractions contained between 19 6 per cent and 34 8 per cent carbohydrate as estimated by the phenol sulphuric acid procedure (*Novotny* 1970) The procedure for the sugar analysis was essentially that described by *Holme et al* (1968) The LPS fractions were treated with 90 per cent v/v aqueous acetic acid at 100° C for 6 hours When the amount of polysaccharide in the fraction had to be determined, xylose was added as an internal standard The hydrolysates were dried by distillation under reduced pressure The residues were hydrolysed in 0 5 N sulphuric acid at 100° C overnight The acid was removed by barium carbonate and the solution concentrated under reduced pressure to about 1 ml The mixtures of aldoses were reduced to the corresponding alditols with an excess of sodium borohydride overnight Excess borohydride was destroyed by 100 per cent v/v acetic acid and boric acid removed as methyl borate by several distillations with methanol under reduced pressure The syrups were dried by distillation under reduced pressure with methanol plus toluene (1 1 v/v) and then fully acetylated with acetic anhydride plus pyridine (1 1 v/v) at 100° C for 10–15 minutes Acetic anhydride was removed by adding water and the resulting solution concentrated to dryness under reduced pressure The resulting mixture of fully acetylated alditol acetates was dissolved in chloroform and analysed by gas liquid chromatography Samples were injected onto an ¼ inch by 5 foot glass column—with 3 per cent ECNSS M (polyestersilicone) as the liquid phase on Chromosorb Q (80–100 mesh) as the solid phase—fitted in a Varian Model 1400 gas chromatograph with a flame ionization detector The injection temperature was 290° C, column temperature 190° C detector temperature 250° C and gas flow (nitrogen) 30 32 ml/min The relative peak areas were determined for the mixtures from the LPS in fractions F3 and F5 and the molecular

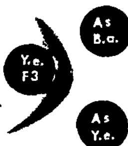
A.



B.



C.



D.



Fig 1 Immunodiffusion Rabbit antiserum (As) to whole cells of *B abortus* (B a) and Y *enterocolitica* type IX (Y e) tested in pairs against fractions 3 (F3) and 5 (F5) from B a (A and B) and Y e C and D

ratios for the constituent sugars calculated from these The different sugars were identified by their retention times and mass spectra (*Bjorndal et al* 1967

RESULTS

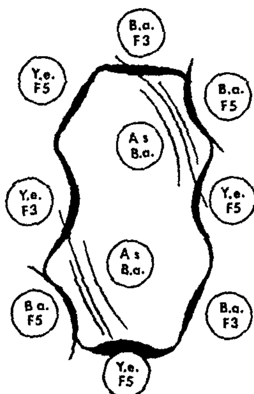
Immunodiffusion Experiments

The aqueous fraction from *B abortus* (B.a F3 gave rise to a heavy precipitate close to the antigen well if tested against *B abortus* B a) and Y *enterocolitica* type IX (Y e) antisera (Fig 1 A) Furthermore, the precipitates showed identity The phenol fraction (B a F5) also gave a line near the antigen well (Fig 1 B) In addition, three lines were found in the reaction with homologous B a antiserum but not with the Y e antiserum

The same main features were shown in the reactions between the aqueous and phenol fractions of Y *enterocolitica* type IX (Y e F3 and Y e F5 respectively) and correspond-

8*

A.



B.

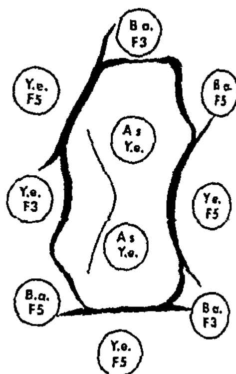


Fig 2 Immunodiffusion Fractions 3 (F3) and 5 (F5) from *B abortus* (B a) and *Y enterocolitica* type IX (Y e) tested against rabbit antiserum (AS) to whole cells of B a (A) and Y e (B)

ing antisera, a heavy precipitate line close to the antigen well was obtained (Fig 1 C and D) In this case, one additional weak precipitation line was formed between the aqueous fraction and homologous antiserum

The precipitates close to the antigen wells show reactions of identity (Fig 2 A and B) Both fractions of Y e gave rise to identical reactions with the aqueous fraction of *B abortus* (B a F3) and a reaction of partial identity with its phenol fraction (B a F5) if tested against the heterologous antiserum (As B a) (Fig 2 A) Similarly, the two fractions of B a yielded reactions of identity with the

TABLE 1 *Relative Proportions of Monosaccharides in the Lipopolysaccharide (LPS) Fractions F3 and F5 of* B abortus *(B a) and* Y enterocolitica *type IX (Y e)*

LPS fractions	Per cent polysaccharide in LPS	Mannose	Galactose	Glucose	Heptose
B a F3	6	0 2	1 0*	5 6§	ND†
B a F5	13	0 7	1 0	1 0	ND
Y e F3	8	ND	1 0	2 9	2 6
Y e F5	14	ND	1 0	1 5	1 8

* Approximate molar ratios of sugars are given relative to galactose taken as 1 0

§ Partly derived from a glucan

† Not detected

aqueous fraction of *Y enterocolitica* type IX (Y e F3) and a reaction of partial identity with its phenol fraction (Y e F5) if tested against the heterologous antiserum (As Y e) (Fig 2 B) The position of the lines near the antigen well indicates that the antigens diffuse slowly, probably because LPS are high molecular complexes

Attempts to identify the chemical nature of the antigenic determinants were made by subjecting the fractions to heat, proteolytic and lipolytic enzymes, and periodate oxidation

Heating to 100° C and treatment of the fractions with a *Streptomyceus griseus* protease left the heavy precipitate near the antigen well unaltered However, the additional precipitates formed between the untreated B a F5 fraction and the homologous antiserum (3 lines Figs 1 B, 2 A) and between the untreated Y e F3 fraction and the homologous antiserum (1 line, Figs 1 C, 2 B) had disappeared, indicating that they were proteinaceous Treatment of the fractions with two different lipases did not change the precipitation patterns formed

If the antigens had been subjected to periodate oxidation the precipitates near the antigen wells were not formed This indicates the carbohydrate nature of the cross reacting antigenic determinants

Sugar Analysis

In order to elucidate the monosaccharide composition of the fractions, a qualitative and quantitative analysis was made using gas liquid chromatography of peracetylated alditol acetates (Table 1)

The carbohydrate content in the fractions was estimated with xylose as internal standard (xylose was shown not to be present in these LPS) The LPS both from the aqueous and from the phenol phase were found to contain relatively small amounts of polysaccharides, the concentrations varying from 6 to 14 per cent The highest amounts were found in the phenol phase The monosaccharides found in the LPS of B a were mannose, galactose and glucose Heptose and aminosugars were not found Galactose and glucose were found in equimolar concentrations (most of the glucose in the F3 fraction was by methylation analysis shown to derive from a glucan, unpublished data), whereas the mannose content was lower The LPS from Y e contained galactose, glucose and heptose Mannose and aminosugars could not be found Glucose and heptose were found in almost equimolar concentrations

DISCUSSION

The present study shows that the cross reacting structures in *B abortus* and *Y enterocolitica* type IX are most probably found in the LPS of the cell envelope

The extraction with hot phenol-water is one of the most commonly used methods by which to extract LPS from gram negative bacteria LPS have furthermore been isolated both from the aqueous and the phenol phases This is due to the partition of hydrophilic and hydrophobic material, respectively The overall composition of the aqueous (F3) and phenol (F5) phases within each species was similar (*Hurvell* 1973)

LPS are mostly found as high molecular complexes with particle weights of several millions (*Shands* 1971) In accordance with this and also because of their low solubility in water, the precipitates against homologous and heterologous antisera in gel diffusion tests were found near the antigen wells (Figs 1 and 2) It is therefore probable that these heavy lines were the results of an interaction between antigenic determinants on the LPS and their antibodies The F3 and F5 fractions showed reactions of identity In spite of differences in the partition coefficient between water and phenol the LPS may contain common antigenic determinants

In the genera of *Enterobacteriaceae*, i e *Salmonella*, *Shigella* and *Escherichia*, in which thermostabile serological cross reactions have been found, these have been shown to be located in the polysaccharide part of the LPS That this is also valid for the

cross-reaction between B a and Y e was based on the following facts

Periodate oxidation, which splits a carbon ring with two vicinal hydroxyl groups, was found to destroy the precipitating ability of the antigens Periodate oxidation has, however, also been shown to have an effect on proteins (*Goebel & Perlmann 1948*) The possibility that the periodate treatment should have influenced a proteinaceous structure was rendered less likely, since the cross-reacting antigens were unaffected by heating to 100° C for 20 minutes and by treatment with a proteolytic enzyme Furthermore, the nitrogen content of the preparations was low, varying between 0 7 and 3 4 per cent

Facts that argue against the idea of having the cross-reacting determinants in the lipids of the LPS are that two lipases have no effect on the cross reactivity and that the lipid part of LPS generally shows low immunogenic activity (*Galanos et al* 1971) Moreover, the cross reactivity should not be expected to be limited to smooth forms of *Brucella* and to serotype IX of *Y enterocolitica* (*Hurvell et al* 1971, *Hurvell* 1972), but should also be observed with rough mutants, i e *B canis* and *B ovis*, and other serotypes of *Y enterocolitica*

The LPS from both B a and Y e had a low content of carbohydrate (Table 1) The maximal amount was estimated to 14 per cent in the Y e F5 fraction LPS from *Y pseudotuberculosis* were also found to have a low carbohydrate content (*Brubaker et al* 1972) By way of comparison, the LPS of rough mutants of *Salmonella typhi-murium* contained 12 to 20 per cent The strains investigated were, however, smooth The evidence of smoothness was obtained by the oblique light technique (*Henry* 1933), acriflavine test (*Braun & Bonestell* 1947) and (agglutinating) stability in saline In the qualitative and quantitative sugar analyses, the F3 and F5 fractions were found to differ in the relative molar proportions of the components found (Table 1) Either the bacteria synthesize different LPS or the fractions contained impurities Attempts to obtain more extensively purified fractions for the sugar analyses are in progress The B a and Y e LPS have glucose and galactose as common monosaccharide constituents In addition the LPS of B a contained mannose and that of Y e heptose Other investigators have described the presence of rhamnose and heptose in *B abortus* using thin layer chromatography (*Kellerman et al* 1970) None of these components, for which the analytical procedure was suitable, were found in our analyses

Nothing is known about the structure of the O-antigens in *Brucella* and *Yersinia*, e g whether they have a basal core and O specific side-chains In Brucella, rough mutants (*B canis* and *B ovis*) do not cross react with *Y enterocolitica* type IX (*Hurvell* 1972) This makes it probable that the cross reactivity in *B abortus* resides in an O specific side-chain Attempts are in progress to determine the structure of the cross reacting polysaccharide side-chains in *B abortus* and *Y enterocolitica* type IX using methylation analysis

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CELL DIVISION AFTER INHIBITION OF DNA AND PROTEIN SYNTHESIS IN *NEISSERIA MENINGITIDIS*

1 Blockage of Division in Synchronized Populations by Hydroxyurea and Chloramphenicol

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Synchronous cultures of *Neisseria meningitidis* Strain M1 have been used to investigate the age at which cell division becomes independent of DNA replication and protein synthesis A transformable variant of the strain was compared with a non transformable one These variants differ with regard to chromosome replication presumably by having different origin and direction of replication Inhibition of DNA replication with hydroxyurea prior to the completion of the cycle of chromosome replication blocked cell division as measured by viable counts in both variants Inhibition of protein synthesis with chloramphenicol alone, as well as in combination with hydroxyurea, blocked cell division until a definite region (or locus) had been replicated This region, which is probably the same in both variants is located close to or at the origin (or terminus) postulated for the chromosome of the non transformable variant The replication of this region also corresponds very nearly in time with the start of cell division in both variants The evidence presented suggests that the replication of a distinct chromosomal region triggers cell division and that protein synthesis simultaneously with this event is also required But this region is not necessarily identical with the origin or terminus of the chromosome

Chromosome replication has been compared in variants of the *Neisseria meningitidis* Strain M1 which were competent (cp^+) in DNA transformation and in variants which were incompetent (cp^-) The change from competence to incompetence was followed by a change in the replication origin as well as in the direction of replication (7, 10, 12) The timing of cell division has also been related to chromosome replication in synchronized cultures of the two variants (12) In these experiments, chromosome replication was examined by enumeration of mutants induced by nitrosoguanidine according to the principles described by *Cerda-Olmedo et al* (1) The experiments show that the timing of cell division relative to the chromosome replication cycle is different in the two variants There is indication that the start of cell division is related in time to the replication of a distinct region or locus which is located rather close to the origin (or terminus) of the incompetent variant, whereas it is well displaced from the origin suggested for the competent variant (12)

In this study synchronized cultures of *N meningitidis* Strain M1 were used to investigate the age during the chromosome

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replication cycle at which cell division becomes independent of DNA replication and of protein synthesis Again advantage was taken of the difference observed with regard to chromosome replication in the transformable and the non transformable variant (7, 10, 12)

MATERIALS AND METHODS

Strains The following auxotrophic mutants from the wild type Strain M1 of Group B were used M1 6 *his pro* and M1 8 *his arg* Variants which were competent in transformation were indicated by the symbol cp^+ and incompetent ones by cp^- The growth requirements as well as competence in DNA transformation were controlled as described previously (11, 12)

Media Blood agar plates and Heart Infusion Broth (HIB Difco) agar plates were used as solid complete media Fluid complete medium was Brain Heart Infusion Broth (BHI, Difco) The basal media were those previously described (6 12)

Growth Growth was followed by measuring absorbancy (11, 12) In most experiments the cultures were grown with shaking in flasks provided with a side arm for reading of the absorbancy Viable counts were performed in samples taken at the desired times The samples were rapidly cooled in ice water for 15 sec Colony forming units were determined by the plating of appropriate dilutions on blood agar plates with the average of two or four plates of viable counts (12)

Synchronization of the growth Chromosome alignment by means of chloramphenicol treatment and synchronization of the growth after release from the inhibition was performed according to the method used before (10 12) A 20 ml amount of BHI was inoculated with the test mutant and incubated on a shaker at 37° C In the exponential phase (approximately at A = 0 300) chloramphenicol was added in a final concentration of 2 5 µg/ml and the culture was incubated with shaking for another two hours at 37° C Subsequently the cells were harvested by centrifugation (20 min at 2500 × g) and resuspended in 2 ml saline This suspension was used to inoculate 100 ml BHI prewarmed to 37° C The culture was next incubated with shaking on a water bath The growth was followed in samples taken at regular intervals usually every 10 minutes Inhibitors were added at the desired times as registered in the individual experiments. Hydroxyurea was used in the concentration 0 015 M whereas the chloramphenicol concentration was 2 5 µg ml (8 9

Mutagenesis Relative numbers of Str r mutants during synchronous growth were measured after mutagenesis with nitrosoguanidine as previously described (10 12)

Chemicals Hydroxyurea and chloramphenicol were obtained from Sigma Chemical Company, St Louis Mo The hydroxyurea stock was a 1 M sterile solution in water Chloramphenicol was dissolved in 50 per cent ethanol at 1 mg/ml

RESULTS

Inhibition with hydroxyurea If completion of a round of replication is a necessary and sufficient condition of DNA synthesis for cell division, the inhibition of DNA synthesis before this event should inhibit division, whereas after this event it should not (2, 4) Hydroxyurea, which is bacteriostatic in *E coli* seems to inhibit DNA synthesis in concentrations which do not affect RNA synthesis or protein metabolism (14, 15) Hydroxyurea has also been used as an inhibitor, preferentially of DNA synthesis in *N meningitidis* (8 9)

Synchronized cultures of the *N meningi-*

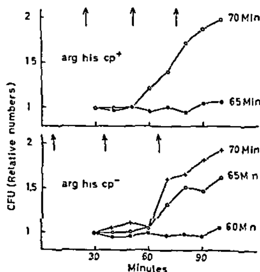


Fig 1 Inhibition of cell division by hydroxyurea Synchronous cultures of competent (cp^+) and incompetent (cp^-) variants of *N meningitidis* Strain M1 were incubated with shaking at 37° C. At the times indicated 0 015 M hydroxyurea was added Number of colony forming units (CFU) was determined by plate-counts. Vertical arrows indicate the peaks of Str r mutants after mutagenesis with nitrosoguanidine (10 12)

TABLE 1 Age of Synchronous Cultures of N meningitidis *Strain M1 at which Cell Division becomes Independent of Inhibition by Hydroxyurea and Chloramphenicol*

Test microbe	Time at which the inhibitor was added		Cell division after the addition of inhibitor*		
	In minutes	In replication units§	Hydroxyurea	Chlor amphenicol	Hydroxyurea + Chlor amphenicol
M1–8 *arg his cp*⁺	45	1 80	—	—	—
	50	2 00	—	—	—
	55	2 20	—	—	—
	60	2 40	—	+	+
	65	2 60	—	+ +	+ +
	70	2 80	+ + +	+ +	+ +
	75	3 00	+ + +	+ + +	+ + +
M1–8 *arg his cp*	45	1 50	—	—	—
	50	1 66	—	—	—
	55	1 83	—	—	—
	60	2 00	—	—	—
	65	2 16	+ +	+	+
	70	2 33	+ + +	+ +	+
	75	2 50	+ + +	+ +	+ + +

* — to + + + indicates various degree of cell division (Cf Fig 1, Fig 2 and Fig 3)

§ Replication unit is the average distance between the successive peaks of Str r mutants after mutagenesis with nitrosoguanidine (12)

tidis Strain M1 were obtained as described above Sequential mutagenesis was first checked in such cultures by mutagenesis with nitrosoguanidine and registration of the peaks of Str r mutants The results were in agreement with those previously reported, with the peaks of mutants appearing at almost exactly the same times (12) The distance between two successive Str-r peaks has been called the "replication unit"

Similar cultures were next treated with hydroxyurea at various ages, and cell division was measured by colony counts Table 1 shows that inhibitor added at all times up to 65 min completely stopped cell division in the *cp*⁺ variant, and up to 60 min in the *cp* one Fig 1 shows that the point of time at which cell division becomes independent of hydroxyurea inhibition is rather distinct No attempts were made to obtain a further resolution than that obtained with 5 min intervals These findings could be very exactly reproduced in subsequent experiments

In Fig 4 the times at which cell division becomes independent of hydroxyurea have been related to the replication maps previously constructed (7, 9, 10, 12) The peaks of Str r mutants served as a common coordinate in the two series of experiments It is seen that the time at which cell division becomes independent of hydroxyurea coincides rather well with the replication of the origin of replication suggested for the *cp*⁺ as well as for the *cp* variant, in the way that in both, two entire "replication waves" have been completed

Inhibition with chloramphenicol It has been postulated that in *E coli* the arrival of the replication point at the terminus of the chromosome is followed by a series of events which terminate in division (5) In other microbes like *Bacillus subtilis* cell division does not seem to be triggered by the completion of rounds of chromosome replication (3) If protein synthesis is involved in such "triggering" it should be required at least until the completion of the chromosome Therefore, we would expect that cell division

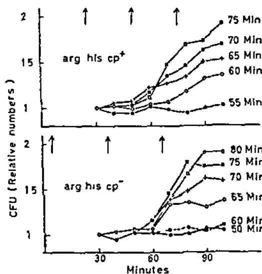


Fig 2 Inhibition of cell division by chloramphenicol Synchronous cultures of competent (cp^+) and incompetent (cp^-) variants of *N meningitidis* Strain M1 were incubated with shaking at 37° C At the times indicated 2 5 µg chloramphenicol were added per ml Number of colony forming units (CFU) was determined by plate-counts. Vertical arrows indicate the peaks of Str r mutants after mutagenesis with nitrosoguanidine (10, 12)

should be blocked by inhibitors of protein synthesis for at least as long as it is blocked by inhibitors of DNA synthesis

The time after release from prolonged chloramphenicol treatment at which cell division becomes independent of inhibition by a new addition of chloramphenicol has been shown in Table 1 In the cp^+ variant division is completely inhibited at all times up to 55 min, whereas in the cp^- one, it is entirely blocked up to 60 min Fig 2 shows the growth curves obtained with inhibitor added just before and immediately after the critical points of time It is seen that a family of curves is obtained, representing increasing degree of cell division The period from no division to complete division expands over more than 15 min It is also seen that the actual period corresponds rather well with that in which cell division does actually take place in the synchronized population (12)

In Fig 4 the results have been presented in replication units and related to the replication map The age at which cell division becomes independent of chloramphenicol corresponds well with that at which it becomes independent of hydroxyurea in the cp^- variant, *i e* just after the replication of the region between *arg* and *his* in which the origin of replication has been located in this variant But in the cp^+ variant, cell division becomes independent of chloramphenicol around 10 min *before* it becomes independent of hydroxyurea This age does not at all correspond to the replication of the terminus (or origin) of replication which has been located at a place close ahead of the *pro* locus (7, 10, 12) But it follows just after the completion of the replication of the region between *arg* and *his* just as it does in the cp^- variant

Inhibition with hydroxyurea plus chloramphenicol In the experiments described above cell division has been measured by the registration of colony forming units Since

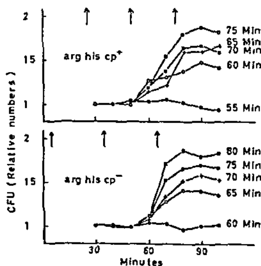


Fig 3 Inhibition of cell division by simultaneous addition of hydroxyurea and chloramphenicol Synchronous cultures of competent (cp^+) and incompetent (cp^-) variants of *N meningitidis* Strain M1 were incubated with shaking at 37° C At the times indicated 0 015 M hydroxyurea plus 2 5 µg/ml chloramphenicol was added Number of colony forming units (CFU) was determined by plate counts. Vertical arrows indicate the peaks of Str r mutants after mutagenesis with nitrosoguanidine (10, 12)

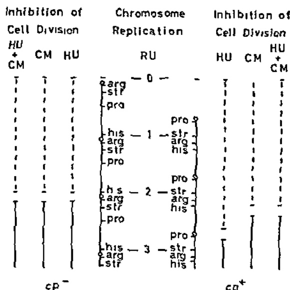


Fig 4 Time chart showing the ages at which cell division in synchronized cultures becomes independent of inhibition by hydroxyurea (HU) and chloramphenicol (CM) The replication maps have been constructed according to previous data (7, 10, 12) Replication unit (RU) is the average distance between successive Str r peaks after mutagenesis with nitrosoguanidine (1 10 12) The origins of replication suggested for the competent (cp^+) and the incompetent (cp) variants of the *N meningitidis* Strain M1 have been indicated with the symbol O

protein synthesis with no concurrent DNA synthesis might result in cell division with non viable progeny, a series of experiments comprising simultaneous addition of hydroxyurea and chloramphenicol was performed

The age at which cell division becomes independent of a combined block by both inhibitors has been shown in Table 1 and in Fig 3 In Fig 4 the results have been recorded in replication units It is seen that a simultaneous addition of the two drugs gives results which are virtually indistinguishable from those obtained with chloramphenicol alone It is also noted that when chloramphenicol is present in synchronous cultures of the cp^+ variant the hydroxyurea block is obviously moved from 65 min to 55 min That is, to the age at which the region between *arg* and *his* has just been replicated

DISCUSSION

Sequential mutagenesis of several genetic markers by means of nitrosoguanidine in synchronized cultures of the *N meningitidis* Strain M1 demonstrates a "replication wave" starting from the origin of replication, and ending with the next one (10,12) The interval between two successive peaks of mutants has been coined the "replication unit" (12) This work confirms the previous finding that the division time in synchronous cultures such as established, is twice the interval between two successive peaks of mutants (12) This division time corresponds well with the generation time in exponential cultures grown in the same medium, as well as to the doubling of the cell mass (12) In accordance with the theories of *Cerdá-Olmedo et al* (1) it may be assumed that the "replication wave" indicates the replication of the entire chromosome and that cell division (and separation into colony-forming units) requires two successive replications in the diplococcus *N meningitidis* The "replication wave" takes 25 (cp^+) to 30 (cp) minutes at 37° C (12) Assuming that the replication speed is approximately as in *E coli* which needs not less than 40 minutes for one replication cycle (2, 5), the chromosome of *N meningitidis* should be at most 60-70 per cent of the *E coli* chromosome *Kingsbury* (13) has indeed found that the *N meningitidis* genome size is only around 40 per cent of that of *E coli*

Since the interpretation of the "replication wave" in the Strain M1 of *N meningitidis* is not entirely clear at the present stage (12), these discussions are based on the provisional, descriptive theory that two such waves constitute one "replication cycle"

The stage in the division cycle after which inhibition of a synthetic process no longer inhibits cell division has been called a "transition point" (4) During the present experiments hydroxyurea was used as an inhibitor [illegible] synthesis and chloramphenicol of [illegible] synthesis Although previous work [illegible] this is the case in *N meningi*

tidis (8, 9, 10) it should be kept in mind that the chemicals possibly could have other effects

With these reservations, the transition point for protein synthesis seems to be at the age of the second "replication wave" when a locus or region near the *his* locus is replicated (Fig 4) The region is located close to or at the origin (or terminus) postulated for the chromosome of the *cp* variant (7, 10, 12) It also represents the age of the synchronized culture at which previous as well as the present experiments show that cell division actually starts (12)

The transition point for DNA synthesis seems to be at the age when two chromosome "replication waves" (or one "replication cycle") have been completed in the *cp* as well as in the *cp*⁺ variant (Fig 4) The completion of a cycle of chromosome replication has been found to be a transition point in a variety of organisms (4) The present results are consistent with this concept when we consider the experiments with hydroxyurea as the only inhibitor But when chloramphenicol is used in addition to hydroxyurea, the transition point of the *cp*⁺ variant is moved to an age more than 10 minutes earlier, just as it is when chloramphenicol is used alone (Table 1 Fig 4) Thus chromosome replication is in fact only required until the region corresponding to that situated close to the *his* locus has been replicated in order to commit the cell to division

Protein synthesis without simultaneous DNA synthesis may presumably result in cell division during the period after the cell has been committed But non viable units probably result until a complete replica of the entire genetic material has been synthesized It would thus seem that the regulation of chromosome replication and protein synthesis is uncoupled for a period during the replication cycle of the *cp* variant

The present experiments indicate that in *N meningitidis* Strain M1 the replication of a certain locus or region is essential for the commitment to cell division and that protein synthesis connected with this event is required The actual region seems to be located very close to the origin (or terminus) for chromosome replication in the *cp* variant Thus, the *cp* variant behaves very much like the *E coli* Strain B/r in this respect (2) But the results obtained with the *cp*⁺ variant of the *N meningitidis* Strain M1 also support the view that it is not the completion of a round of chromosome replication *per se* that is the essential event, but the replication of a chromosomal region near or at the terminus (or origin) of the *cp* variant

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CELL DIVISION AFTER INHIBITION OF DNA AND PROTEIN SYNTHESIS IN *NEISSERIA MENINGITIDIS*

2 Residual Division and Increase in Absorbancy in Exponential Phase Cultures After Addition of Hydroxyurea and Chloramphenicol

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A comparison has been undertaken between a transformable (*cp*) and a non transformable (*cp*) variant of the *Neisseria meningitidis* Strain M1 Previous evidence indicates that these variants have different origin and direction of replication Hydroxyurea inhibition resulted in 41 per cent and 43 per cent increase in colony forming units in exponentially growing cultures of the *cp* and the *cp* variants respectively Chloramphenicol exhibited a concentration effect At 2 5 μg/ml exponential increase in colony forming units stopped, but the number increased with 73 per cent in the *cp* variant and with 45 per cent in the *cp* one Concentrations above 10–15 μg/ml permitted no significant residual division The rate of increase in absorbancy was reduced by hydroxyurea and chloramphenicol but there was no significant difference between the two variants The findings have been discussed in relation to the age distribution function for bacterial chromosomes in an exponential population The results support the hypothesis that there are two kinds of proteins involved in cell division The synthesis of one of these is inhibited by low concentrations of chloramphenicol and seems to be triggered by the replication of a distinct region or locus on the chromosome This region is probably located close to or at the origin (or terminus) for the chromosome of the *cp* variant, whereas it is distant from that for the *cp*⁺ one

Most of our present knowledge concerning the relationship between chromosome replication and cell division has been obtained from studies with rod shaped bacteria like *Escherichia coli* and *Bacillus subtilis* (5, 23) Since *it is of interest to know whether the general* traits which pertain to bacilli are also valid for bacteria with an entirely different morphology, experiments were started with the diplococcus *Neisseria meningitidis* (16, 17) During these experiments advantage was taken of a difference that has been observed with regard to chromosome replication in a transformable (*cp*⁺) and a non transformable (*cp*) variant of the *N meningitidis* Strain M1 (11, 12, 13 16)

At the outset, measurements were performed *in order to relate the timing of cell* division to the chromosome replication cycle in synchronous cultures (16) Subsequently, the stages in the replication cycle of such cultures at which division becomes independent of hydroxyurea and chloramphenicol inhibition were made out (17)

The evidence accumulated indicates that the replication of a distinct chromosomal

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region (or locus) triggers cell division, and that protein synthesis simultaneously with this replication is required It also seems that the start of cell division coincides very nearly in time with this event But division is not complete until around 20 minutes later

The actual chromosomal region is located close to or at the origin (or terminus) of the chromosome of the *cp* variant Thus, this variant behaves very much like the *E coli* Strain B/r (2, 3, 8) But the *cp*⁺ variant behaved differently Also in this variant, commitment to division seems to be connected with replication of the same chromosomal region, and with concurrent protein synthesis But there are several lines of evidence indicating that in *cp*⁺ meningococci the origin of replication is located at an entirely different site (11, 12 13, 16, 17) The experiments with *N meningitidis* thus support the view that it is not the completion of a round of chromosome replication *per se* that "triggers" cell division in bacteria behaving like the *cp* variant, but the replication of a chromosomal region near or at the terminus (or origin)

In the present communication the extent of residual cell division and accumulation of cell mass have been measured in exponentially growing cultures of *cp*⁺ and *cp* variants of the *N meningitidis* Strain M1 The experiments were designed in order to obtain an approximation of the critical age beyond which DNA synthesis is not required for cell division The theoretical background for this approach has previously been sketched out in connection with work with *E coli* Strain B/r (2, 3)

MATERIALS AND METHODS

The methodology and experimental manipulations used in this communication were the same as those described in the preceding papers (16, 17) The following is especially relevant to the present report

Bacteria M1 6 *his pro* and M1 8 *his arg* are mutants from the wild type *N meningitidis* Strain M1 of Group B Variants which were competent in DNA transformation were indicated by the symbol *cp*⁺ and incompetent ones by *cp* (16)

Inhibition of growth Inhibition of growth in exponential cultures was tested in growth flasks provided with a side-arm for the reading of absorbancy (15, 16) 11 5 ml Brain Heart Infusion Broth (BHI, Difco) was inoculated from a culture in the exponential phase to A = 0 080 and incubated at 37° C on a shaker When the turbidity had reached A — 0 180 the experiment proper was started (time zero), with reading of absorbancy and counting of colony forming units every 15 or 30 minutes The inhibitor was added after 60 minutes and the measurements continued for another 4 hours Hydroxyurea was used in 0 015 M and chloramphenicol in the final concentration 2 5 μg/ml (17) unless otherwise indicated

Principles of calculation If replication (gene duplication) in a definite locus or region is one condition for cell division the interruption of DNA synthesis before this event should inhibit division whereas after this event it should not If protein *synthesis is also required in connection with this* event, inhibition of that biosynthetic function should have the same effect I e, if the actual 'transition point (17) was located near the terminus of the chromosome, little residual cell division should occur in exponential cultures after the addition of an inhibitor of DNA or protein synthesis since only those cells that have been committed before the addition would divide *But* if the region was located at the origin practically all the cells should undergo division The fraction of the chromosomes in an exponentially growing cell population in which a certain marker has been replicated may be determined from the relative frequencies of genetic markers at different positions on the chromosome The frequency g(X) of a marker X located at X on the chromosome can be obtained from the *marker frequency function* (11 22 26 27)

$$g_n(X) = 2^{n(1 X)}$$

Here n is the average number of *replication* positions per chromosome The value n can be calculated from the distance between replication positions d which is equivalent to 1/n To keep a steady state of chromosome replication in the exponential phase population, cell duplication must

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can be determined in conventional technique during exponential growth The value RP may be measured in independent experiments (16) and used in the calculations according to the theory that the replication speed of each replication point is constant without regard to the length of the cell generation time (18 21)

If the hypothetical locus or region at which cell division escapes from dependence on chromosome

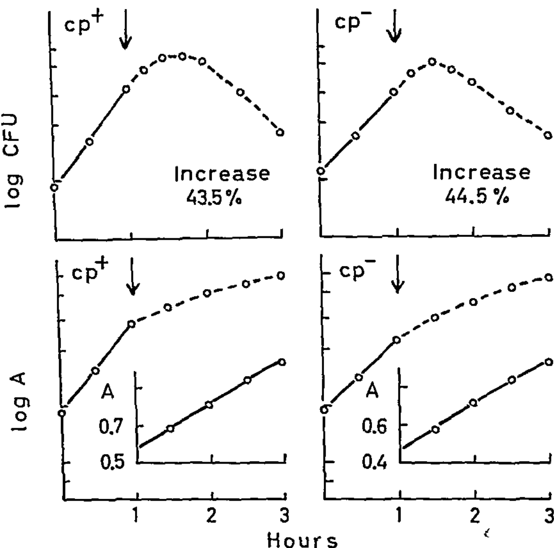


Fig 1 Effect of hydroxyurea 0.015 M on the auxotroph M1 6 *his pro* growing exponentially in BHI medium The time of addition of the inhibitor is indicated by vertical arrows

replication were located at the end (g(1) = 1) no residual division would occur after inhibition of DNA synthesis The value g(X) − 1 may then be taken to indicate the fraction of chromosomes in which the locus X has been replicated This value should correspond to the fraction of cells ("division units") in which cell division may take place

Example showing calculation of the "transition point" The mutant M1-6 *pro his cp*⁺ was blocked with 0.015 M hydroxyurea in the exponential phase

Measurements 1 G = 49.4 min was calculated from measurement of absorbancy in the actual experiment (Fig 1)

2) Replication unit (RU) = 25 min was measured in independent synchronized culture at 37° C after mutagenesis with nitrosoguanidine (16) RU is identical to the average distance between successive peaks of Str r mutants. RP = RU × 2 according to the previous finding that one 'replication cycle' consists of two 'replication waves' (cf discussion)

3) Residual cell division (RCD) = 41.4 per cent was calculated according to counts of colony-forming units (CFU) in the actual experiment (Fig 1) The growth curve was exponential when hydroxyurea was added

Calculation In the marker frequency function

$n - 1/d = 50/49\ 4 = 1\ 012$ and $g(X) - 1 = 0\ 414$ and we get

$$1\ 414 = 2^{1\ 012\ (1-X)}$$
$$x = 0\ 506$$

The 'transition point (17) is accordingly located very close to either the termous of the first or the origin of the second of the two replication waves that constitute the 'replication cycle (16, 17)

RESULTS

Inhibition with hydroxyurea At the outset, a number of experiments were performed to establish a reproducible test system comprising a control of the essential growth parameters The procedure described in the section on methods permits the estimation of the exponentiality of growth, both in terms of absorbancy and in terms of colony forming units The method also ensures that the inhibitor is added to the culture approximately in the middle of the exponential phase No experiment was considered representative unless these requirements were fulfilled

When the mutants M1 6 *his pro* and M1 8 *his arg* were treated with hydroxyurea measurements of colony forming units showed that cell division continued for some time But the growth soon levelled off, and after some time the number of colony forming units started to decrease The increase in absorbancy slowed down after the addition of hydroxyurea, but continued approximately linearly on an arithmetical scale for more than 2 hours whereupon the curve gradually levelled off (Fig 1)

TABLE 1 *Residual Cell Division after the Addition of Hydroxyurea and Chloramphenicol to Exponential Cultures of* Neisseria meningitidis *Strain M1 Calculation of the Points in the Chromosome Replication Cycle at which Division becomes released from the Inhibition*

Mutant analysed	Competence	Expt No	Inhibitor*	Generation time (G) Min	Residual cell division (RCD) %	Distance calculated§ Part of replication period (RP)	Part of replication unit (RU)
6 *his pro*	*cp*	1	HU	49 4	41 4	0 506	1 012
his pro	*cp*	2	HU	53 3	40 8	0 473	0 946
8 *his arg*	*cp*	3	HU	46 6	43 5	0 515	1 028
his arg	*cp*	4	HU	48 9	42 1	0 504	1 008
6 *his pro*	*cp*	1	HU	57 1	42 1	0 515	1 030
his pro	*cp*	2	HU	55 0	42 4	0 532	1 064
8 *his arg*	*cp*	3	HU	56 4	44 5	0 493	0 986
his arg	*cp*	4	HU	54 5	43 2	0 529	1 058
6 *his pro*	*cp*	1	CM	19 4	71 4	0 231	0 462
his pro	*cp*	2	CM	47 4	75 1	0 232	0 464
8 *his arg*	*cp*	3	CM	50 2	73 6	0 204	0 408
his arg	*cp*	4	CM	48 2	72 8	0 239	0 478
6 *his pro*	*cp*	1	CM	52 6	49 1	0 494	0 988
his pro	*cp*	2	CM	54 6	47 2	0 492	0 984
8 *his arg*	*cp*	3	CM	56 4	45 0	0 484	0 968
his arg	*cp*	4	CM	60 5	42 1	0 488	0 976
6 *his pro*	*cp*	1	HU + CM	51 3	70 1	0 214	0 428
8 *his arg*	*cp*	2	HU + CM	48 6	74 1	0 223	0 446
6 *his pro*	*cp*	1	HU + CM	58 4	43 9	0 489	0 978
8 *his arg*	*cp*	2	HU + CM	56 8	41 8	0 523	1 016

* Hydroxyurea (HU) 0 015 M and chloramphenicol (CM) in concentration 2 5 μg/ml

§ Calculated from the function $g_n(X) = 2^{n(1-X)}$ (27) as described in the section on methods

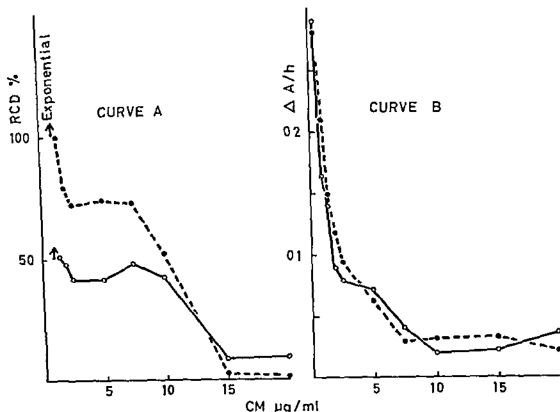


Fig 2 Effect of chloramphenicol concentration on the auxotroph M1 8 *his arg* growing exponentially in BHI medium Curve A shows residual cell division (RCD per cent) and Curve B the rate of increase in Absorbancy (Δ A/h) o——o = *cp* ● --● – *cp*

Residual cell division was next compared in the *cp* and the *cp* variants From Table 1 is seen that the average maximal increase is practically the same in the two variants being 41 4 per cent in four experiments with *cp*⁺ bacteria and 43 1 per cent in four experiments with *cp* ones

The measurements demonstrated no significant difference between the variants in the rate of increase of absorbancy during the two first hours after addition of hydroxyurea (Δ A/h = 0 270 for the *cp*⁺ and Δ A/h = 0 278 for the *cp* variant) The rate of increase started to decrease when the turbidity was around A = 0 800 and the growth finally stopped at approximately A = 1 000 This is very nearly the absorbancy at which growth stops in batch cultures in the same medium when no inhibitor is added (16)

Inhibition with chloramphenicol Chloramphenicol exhibited a clear concentration effect as shown in Fig 2 At concentrations between 2 and 7 5 µg/ml the exponential accumulation of colony forming units stopped, but there occurred substantial residual increase in number But when the concentration was increased to around 15 µg/ml very little if any residual division could be measured No decrease in colony forming units was observed upon the extension of the observation period to more than 3 hours (Fig 3) The effect of various concentrations of chloramphenicol on the rate of increase in turbidity has been shown in Fig 2

Comparison of residual cell division after exposure to concentrations between 2 5 and 7.5 µg/ml of chloramphenicol demonstrated a pronounced difference between the two va-

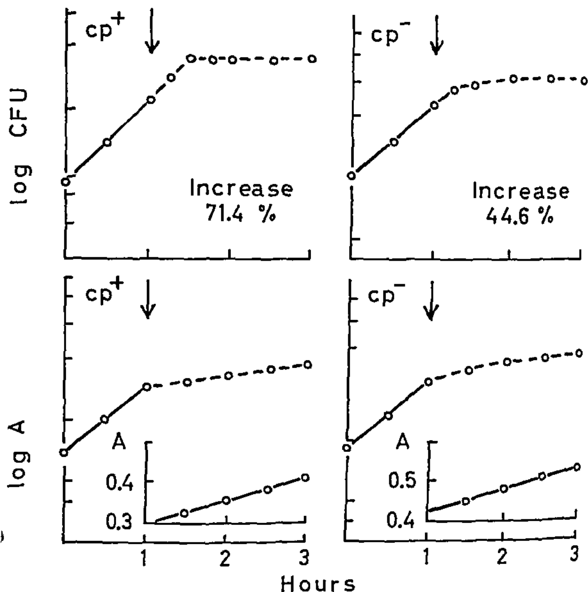


Fig 3 Effect of chloramphenicol 2 5 μg/ml on the auxotroph M1 6 *his pro* growing exponentially in BHI medium. The time of addition of the inhibitor is indicated by vertical arrows

riants of the test microbe (Fig 2, Fig 3) With 2 5 μg/ml the average increase is 73 2 per cent in the cp^{+} mutants, whereas it is only 45 8 per cent in the *cp* ones (Table 1)

Inhibition with hydroxyurea plus chloramphenicol When 2 5 μg chloramphenicol is used per ml in addition to 0 015 M hydroxyurea, there is still some increase in the turbidity (Fig 4) A complete stop is obtained, however, with 15–20 μg/ml chloramphenicol under otherwise identical conditions

The combined addition of hydroxyurea plus chloramphenicol has practically the same effect as chloramphenicol alone on cell division The residual division after the addition of 2 5 μg/ml chloramphenicol and 0 015 M hydroxyurea was average 72 1 per cent in the cp^{+} variant and 42 8 per cent in the *cp* one (Fig 4, Table 1) It is also seen that hydroxyurea has a killing effect during prolonged incubation even in the presence of chloramphenicol

Residual division related to chromosome

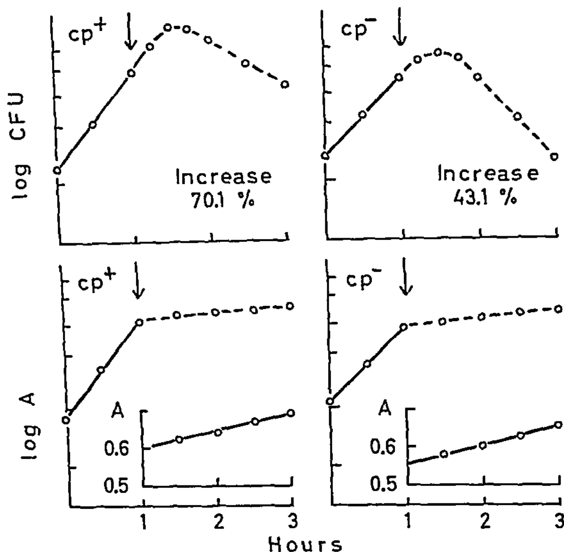


Fig 4 Effect of the addition of hydroxyurea (0 015 M) plus chloramphenicol (2 5 μg/ml) on the auxotroph M1 6 *hu pro* growing exponentially in BHI medium The time of addition of the inhibitors is indicated by vertical arrows

replication The residual growth was next related to chromosome replication according to the principles sketched out in the section on methods Table I shows several experiments comparing the *cp* variants of the test mutants with the *cp* ones These experiments as well as several others, indicate that this "mapping procedure gives very reproducible results for the value of x

The average calculated distances have been presented in Fig 5 and related to the replication maps representing the chromosomes in competent and incompetent variants of the N *meningitidis* Strain M1 (11, 12, 13 16) Apparently, cell division in both variants escapes from hydroxyurea inhibition when one "replication wave" has been completed In the *cp* variant it also seems to escape inhibition of 2 5 μg/ml chloramphenicol at the same time in the replication cycle But in the *cp*⁺ variant cell division is obviously released from chloramphenicol inhibition

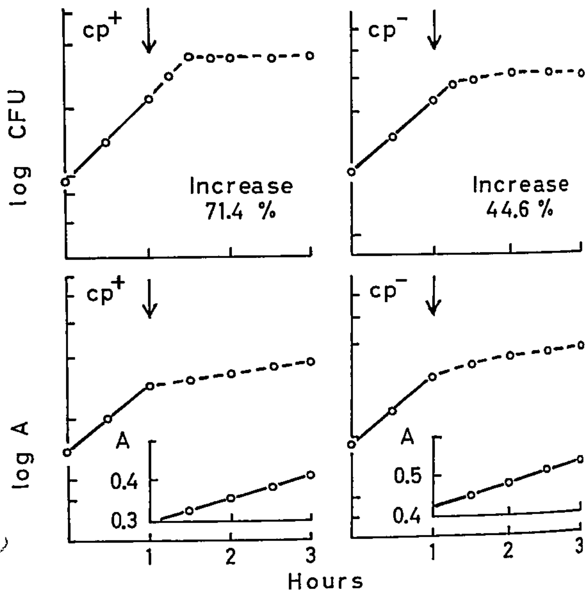


Fig 3 Effect of chloramphenicol 2 5 μg/ml on the auxotroph M1 6 *his pro* growing exponentially in BHI medium The time of addition of the inhibitor is indicated by vertical arrows

riants of the test microbe (Fig 2, Fig 3) With 2 5 μg/ml the average increase is 73 2 per cent in the cp^+ mutants, whereas it is only 45 8 per cent in the cp^- ones (Table 1)

Inhibition with hydroxyurea plus chloramphenicol When 2 5 μg chloramphenicol is used per ml in addition to 0 015 M hydroxyurea, there is still some increase in the turbidity (Fig 4) A complete stop is obtained, however, with 15–20 μg/ml chloramphenicol under otherwise identical conditions

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Residual division related to chromosome

cell division and DNA replication *Lark and Lark* (19) also found that there are at least two proteins that are involved in the regulation of the initiation of DNA synthesis in bacteria One of these seemed to be a structural protein that was resistant to low levels of chloramphenicol This protein could be the one involved in the synthesis of the septum

The present experiments with *N meningitidis* emphasize a concentration effect of chloramphenicol The concentration 2 5 μg/ml is adequate to *stop* cell division, but only after some residual division At higher concentrations (15–20 μg/ml) no significant residual division occurs This indicates that there are two types of protein synthesis involved in cell division with distinct susceptibility to chloramphenicol

Concentration effects of chloramphenicol have also been observed in *E coli* (19), both on protein synthesis *per se*, and on the re initiation of DNA synthesis after amino acid starvation *Lark and Lark* (19) in fact based much of their argumentation for the existence of two proteins involved in the regulation of DNA synthesis on concentration effects

The data from exponential cultures inhibited with hydroxyurea and chloramphenicol point out another pronounced difference between the cp^+ and the cp^- variants of the *N meningitidis* Strain M1 in addition to those reported before (11, 12, 16 17) Residual cell division after the addition of 2 5 μg/ml chloramphenicol is by far higher in the cp^+ variant (ave 73 8 per cent) than in the *cp* one (ave 45 8 per cent) In contrast cell division after the addition of 0 015 M hydroxyurea is very nearly the same in the two variants (ave 41 9 per cent and 43 0 per cent for the cp^+ and the *cp* variants respectively)

As in synchronous cultures (17), a striking change is observed in the effect of hydroxyurea on the *cp* variant when 2 5 μg/ml chloramphenicol is used in addition Residual division is increased from ave 41 9 per cent to 72 1 per cent (Table 1) This supports the previous assumption that chromosome replication is only required until the 'age' at which cell division is released from low level chloramphenicol inhibition Protein synthesis after this commitment, and in the absence of DNA synthesis may lead to division and non viable units in the cp^+ variant, because no complete replica of the entire genetic material has been synthesized (17) The difference between the increase in turbidity and in viable counts after inhibition with hydroxyurea (Fig 1) indicates clearly the possibility of un balanced growth The reason is probably that RNA and protein synthesis proceeds in the absence of DNA synthesis also in *N meningitidis* (24) In *B subtilis* the inhibition of DNA replication results in anucleate cells under conditions similar to those used in the present experiments (5) The synthesis of anucleate cells in the absence of DNA synthesis is known to take place also in certain mutants of *E coli* (9)

The provisional, descriptive model that two "replication waves' constitute one "replication cycle' has been used to relate two parameters of cell division in the *N meningitidis* Strain M1 to the chromosome maps previously constructed (11, 12, 13) The timing of the start of division (16) and the "transition points' for DNA and protein synthesis (17) In order to compare the present data from exponential cultures with those from synchronous ones, they have been discussed in relation to the age distribution function for bacterial chromosomes in an exponentially growing population (22, 26, 27) The approach thus in the principle followed that used by *Clark* (2 3) when he measured residual cell division in *E coli* after inhibition of DNA synthesis, and estimated the age at which the cells escape from the dependence of DNA synthesis in order to divide

The calculations (Table 1, Fig 5) indeed indicate that the "transition points' in exponentially growing cells may be the same as those found in synchronous cultures (17) They also seem to confirm the previous finding that the 'transition points are associated with every second "replication wave" But there is one significant difference The

"transition points" in all experiments occur in the first (for chloramphenicol inhibition in the cp^{+} variant) or at the end of the first (for all other observations) "replication wave" that constitute the 'replication cycle", whereas they occur in or at the end of the second one in synchronous cultures This difference may be a result of the chloramphenicol inhibition used to obtain synchronous cultures (13, 16) The experiments with exponential cultures may thus indicate the "normal" sequence of events There are other observations from the present experiments indicating changes in what we may consider as the normal physiology of the cell during prolonged chloramphenicol inhibition One finding concerns the ratio mass/colony-forming units Since residual cell division is much higher in cp^{+} than in cp variants after chloramphenicol inhibition (Fig 2 and 3) whereas residual mass synthesis is approximately the same, this ratio must be lower in the cp^{+} than in the cp ones when chromosome alignment has been accomplished This change in the physiology must obviously have consequences for the behaviour of the cell after release from the inhibition The lag before the start of chromosome replication is probably one important effect This lag is much longer in the cp^{+} than in the cp variant (16, 17), a finding which of course should be seen in relation to the important finding of *Hanawalt et al* (6) of a relationship between the cellular mass and initiation of chromosome replication It may thus be, that chromosome replication in both variants of the Strain M1 of *N meningitidis* is delayed until the correct ratio mass/colony forming units has been re-established

These experiments indicate that the replication of a certain region or locus on the chromosome either initiates or enhances protein synthesis, and that this protein synthesis which is sensitive to low levels of chloramphenicol is required for the initiation of cell division Two different mechanisms which may be involved in such a phenomenon have been discussed by several authors (7) First, synthesis of protein could actually be enhanced or initiated by the passage of the replication point along the region in question Second, the duplication of the gene could increase the rate of synthesis of the gene product by "gene dosage'

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SEROLOGICAL INVESTIGATIONS INDICATING THE EXISTENCE OF TICK-BORNE ENCEPHALITIS VIRUS FOCI ALONG THE NORWEGIAN COAST

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As a part of investigations attempting to establish the existence and the extent of Tick borne encephalitis viruses in Norway, the haemagglutination inhibition test was carried out using selected bovine sera from areas with *Ixodes ricinus* invaded pastures 14 out of 81 sera were sero-positive with titres higher than 10 Indications are given of an active endemic area in Sunnfjord, as well as virus foci in other localities It is concluded that Tick borne encephalitis, which is not considered a problem in Norway at present, may be more common than previously realized, and that more attention should be given to it both by clinicians and microbiologists

Tick-borne encephalitis virus (TBE) and related arboviruses have previously been investigated, and their extent charted in Sweden (6), Finland (5) and Denmark (4)

In spite of this, these viruses and their associated clinical syndromes have never seemed to evoke any particular interest in Norway, although papers suggesting the existence of TBE in this country have been published recently (1 7 8) On clinical, epidemiological and virus ecological data, these papers confirm that the absence of TBE from Norway would be more peculiar than the opposite situation

One of these papers from 1962 (1) advanced the hypothesis that an endemic area existed in Sunnhordland Out of 27 patients hospitalized with meningo-encephalitis at Fylkessykehuset Stord 12 were suggestive of TBE Recent attempts at virus isolation from ticks (Ixodes ricinus) collected in the localities mentioned were not successful (9)

It has been reported that in endemic areas in Czechoslovakia, cattle grazing on tick invaded pastures are excellent indicators of the existence of virus foci (3) in some localities, 20–30 per cent of the animals were found to be sero-positive

This paper reports the results of a serological investigation of TBE-antibodies in bovine sera collected from known tick invaded localities in Norway

MATERIAL AND METHODS

Bovine Sera

A circular containing information about certain aspects of TBE was distributed to veterinary surgeons practising in known tick invaded areas along the Norwegian coast At the same time these veterinary surgeons were requested to draw blood specimens from cattle belonging to farms which had manifest tickinvasions in the pastures During the following months, specimens were received from

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13 veterinary surgeons while 3 others gave information that ticks no longer represented a problem in their areas because of pasture cultivation etc

Specimens from 81 bovine individuals were received and included in the investigation

As soon as the specimens arrived at the laboratory, the sera were prepared, heated at 56° C for 30 min and stored at -20° C until tested

Positive Control-Serum

This was a convalescent serum from the only verified case of TBE occurring in Norway, a woman (K M) hospitalized at Haukeland sykehus, Bergen, with meningitis serosa and paresis facialis This serum had previously been found to be positive with a titre of 80, by the haemagglutination inhibition test (HAI) The serological diagnosis had been confirmed by Prof *N Oker Blom*, Helsinki, Finland

Negative Control-Serum

For this purpose the serum of a cow which was born and had lived in an area not invaded by ticks was employed

Haemagglutinating TBE Antigen

By the courtesy Professor *D Blascovic* and Dr *M Grestkova*, a sucrose acetone (SA) haemagglutinating TBE antigen was provided by the Institute of Virology, Slovak Academy of Sciences, Bratislava At the time the HAI test was performed, using chicken erythrocytes, this antigen had a titre of 1600

Haemagglutination Inhibition Test (HAI Test)

This was performed as described by *Clarke and Casals* (2) Both dilution of sera and the execution of the final test were carried out with micro-dilution equipment in disposable plastic plates (Cooke Engineering Co , Alexandria, Virginia)

Clarke and Casals recommended 4-8 HA units of antigen in the final test In this investigation the antigen was used in a dilution of 1/150, giving a little more than 10 HA units This was in order to reduce the sensitivity of the test and by is means secure more specific results

RESULTS

Some of the sera showed unspecific agglutination in the serum controls even after extensive absorbtion with chicken erythrocytes, and consequently could give no conclusive results

The positive control-serum showed inhibi-

TABLE 1 *Summary of Relevant Data on Bovine Individuals Sero Positive for Tick Borne Encephalitis*

Residence of veterinarians participating	Total number of specimens	Number of sero-positive individuals	Residence of the sero-positive individuals, and serum titres* of the same	
Sjoholt	10	1	Sjoholt	20
Sande in Sunnfjord	19	7	Bygsjord	20
			Bygstad	20
			Bygstad	20
			Rugdaland	20
			Sande	320
			Viksdalen	40
			Viksdalen	160
Brekke	6	1	Brekke	40
Norheimsund	2	1	Torviksbygd	20
Fikelandsosen	6	1	Olve	80
Rosendal	13	1	Heroysund	20
Olen	9	1	Vikedal	160
Lyngdal	2	1	Herad	20

* Titre i e the reciprocal value of the highest serumdilution giving a total inhibition of haemagglutination

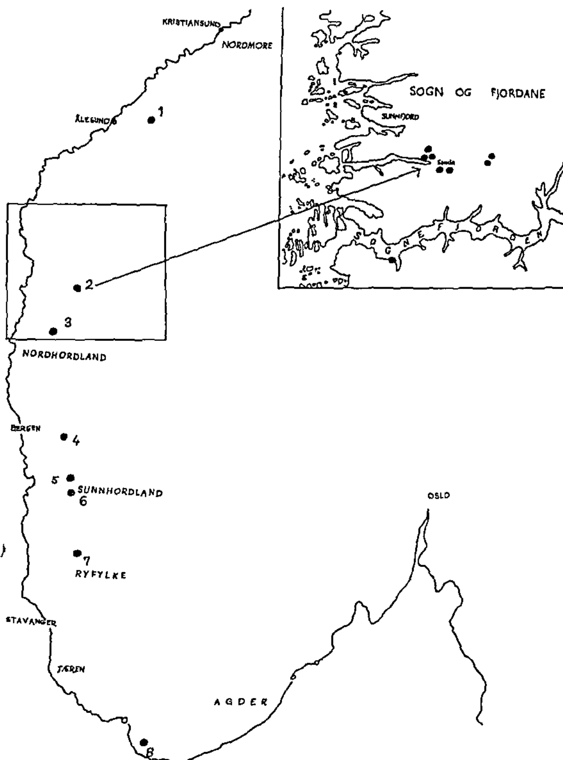


Fig 1 A Each dot is representing one sero-positive bovine individual except in case 2 The situation in this locality is more explicitly illustrated in Fig 1 B The dots are situated at 1 Sjoholt 2 Sande in Sunnfjord, 3 Brekke 4 Torvikbygd 5 Ølve, 6 Herøysund 7 Vikedal 8 Herad
B A more detailed illustration of the distribution around Sande in Sunnfjord One dot represents one sero-positive bovine individual

tion at a dilution of 1/20 When earlier tested with 6 HA units of antigen, it was positive at dilutions 1/80–1/160

The negative control serum never showed any inhibition of haemagglutination

14 out of the 81 bovine sera tested during this investigation were sero positive for TBE with titres higher than 10 (The agglutinating sera were considered negative) *This means that 17 7 per cent of the animals tested had antibodies against TBE*

The geographical distribution of positive individuals along the coast of Norway is shown in Fig 1 A Fig 1 B is a more explicit map showing the distribution of sero-positive cattle around Sande in Sunnfjord

The numbers of tested specimens from each locality and the titres of each sero positive individual are shown in Table 1 In this table, the figures for the area around Sande in Sunnfjord should be specially noticed *7 out of 19 animals are sero positive This represents a percentage of 36 3 per cent*

DISCUSSION

The results presented here are considered specific because of the following facts 1 The sensitivity of the test system was intentionally made very low 2 For this reason a known positive serum which had given a titre of 80-320 in other laboratories was, under the conditions employed here, positive with a titre of 20 3 In spite of the factors mentioned above, only sera giving full inhibition at dilutions greater than 1/10 (which in practice, means ≧20) were considered positive

However, as HAI is a group-specific test, nothing definite can be stated about the exact type (or types) of virus provoking the serological results reported here This investigation shows that viruses related to Tick-borne encephalitis virus by no means, can be considered a curiosity in Norway Previously proposed hypotheses based on clinical, epidemiological and virus-ecological data have indicated that there may exist virus foci of rather high activity along the Norwegian coast The results obtained in this study, however, offer for the first time, virological support to these suggestions

Since the number of specimens tested was very low, this investigation does not permit any conclusions to be drawn regarding the total frequency of seropositive bovine individuals, and the strength of the virus foci in the various areas The situation in Sunnfjord gives the impression of an endemic area, with virus-foci of higher activity than in previously charted areas in Czechoslovakia (3)

Some of the tested sera contained antibodies with a rather high titre (320, 160, 80), while on the other hand the convalescent serum of a human TBE patient had a lower titre under the test conditions employed than previously evaluated High titres may be interpreted either as a sign of recent infection or they may be the result of reinfection In both cases an indication is given of virus foci which have been active up to the present time

Any connection between the occurrence of cattle sero-positive for TBE in a particular area and the probability of human patients suffering from the virus disease cannot easily be calculated, and unfortunately neither human or bovine specimens from the suspected endemic area in Sunnhordland were available The results of this investigation should however, encourage further work in this field and it should urge medical doctors and hospital staffs to take TBE into consideration whenever patients with meningo-encephalitis are encountered

Further investigations which should give some answers to the questions provoked by this work, are in progress

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The author is grateful to Drs. *D Blaskovic* and *M Grešíková* for providing the antigen He also wishes to thank the veterinary surgeons who made this investigation possible by collecting specimens

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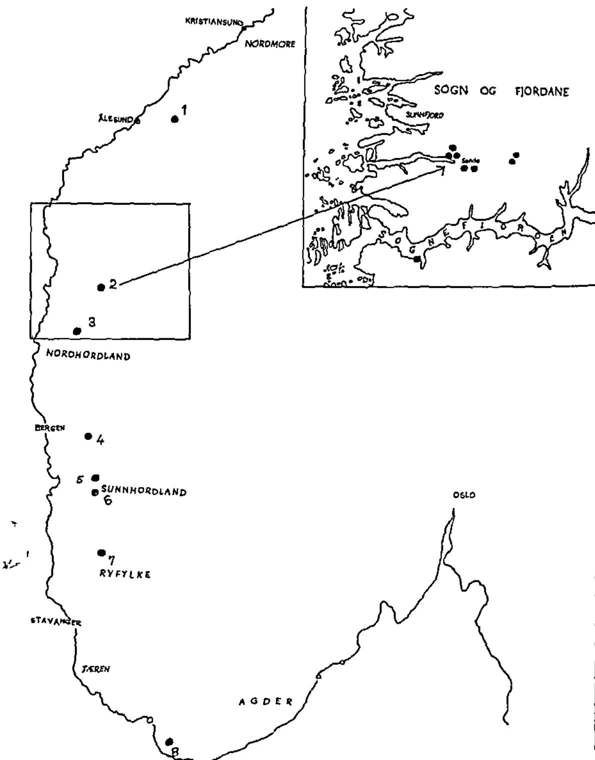


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REQUIREMENTS FOR ANTIBODY IN THE IMMUNE COMPLEX-INDUCED PLATELET AGGREGATION REACTION

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Some properties of the antibodies involved in the immune complex induced platelet aggregation (Pl A) reaction were analyzed in the present study Rabbits were immunized with a hapten protein conjugate (NIP chicken globulin) and the primary response (3 week) IgG was fractionated The Pl A reactivities of these fractions were found to correlate with the titres of haptenated phage inactivation (HPI) when the values were adjusted to represent equal antibody concentrations (measured by hapten binding capacity) The minimum amount of primary response anti NIP IgG detectable with the Pl A method was estimated to be between 1 7 and 14 6 μg/ml Complement fixing antibodies were detected with approximately similar sensitivity The titres of lightly haptenated phage inactivation did not correlate to the Pl A reactivities suggesting that the role of very high affinity antibodies is not critical to the Pl A reaction The affinity requirements of the PlA method was concluded to be close to the standard HPI method (heavy coupling) but considerably lower than that of the HPI method with lightly coupled phage

Antigen antibody complexes can be detected by the platelet aggregation (Pl A) test developed by *Penttinen and Myllylä* (21) The method, based on interaction of small size (soluble) antigens and their corresponding antibodies on fresh human platelets, resembles complement fixation (CF) by antigen antibody complexes in certain aspects The first steps in the sequence of the reactions (combining of antigen and antibody and subsequent changes in the Fc parts of the antibody molecules) appear similar in both CF and Pl A (10) However, only the IgG class of antibodies has been shown to be Pl A reactive (12, 23 25) Also the active site for platelets in the Fc fragment of an IgG molecule seems to differ from the active site for complement (10) The indicator reaction (viable human platelets as target cells for immune complexes, not requiring the presence of complement (10)), differs also essentially from other serologic agglutination techniques

The Pl A technique proved to be very sensitive in detecting antigen ($NIP_{40}BSA$, see Methods) the smallest amount measurable was 1 6 ng/ml (23) and the test appeared to be about 100 times more sensitive than the CF technique in this respect Compared to the CF technique the ability of the PlA test to detect hyperimmune anti NIP antibody was also relatively good 0 3 μg/ml was detected The CF technique was about 16 times less sensitive for measurement of antibody

The Pl A method has been applied to the

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study of several viral antigens and antibodies (8, 11, 12, 16, 18–22, 24) Despite the apparent sensitivity of the Pl A technique, the CF method was superior in detecting 'early' convalescent antibodies (1–3 weeks after onset of infection) in primary herpes simplex (18), varicella (16) and measles virus infections (19) The Pl A test gave, on the other hand, higher titres than CF in recurrent herpes simplex, herpes zoster and subacute sclerosing panencephalitis (a chronic infection by measles virus) In the earliest primary response sera CF-reactive IgM may account for these differences but in later sera (2–3 weeks) other differences within the antibody population are thought to predominate

Variation in antibody class, content and affinity are evidently factors that contributed to the inconsistent and usually poor Pl A reactivity of "early" convalescent sera Unfortunately viral antigens and antibodies are usually very complex and antibody affinity cannot be evaluated quantitatively by current methods A simple antigen, preferably a haptenated protein, is required, the antibody (of only one class) should be purified and the molar concentration of the antibody should be known

Inactivation of haptenated phage (HPI) by specific antiserum (13) is considered to be the most sensitive antibody assay available An amount of 0 5 ng/ml of primary response anti NIP IgG and 0 02 ng/ml of late IgG can be detected (26) The HPI titres correlate roughly to the amount of antibody which can be estimated as described by *Sarvas and Mäkela* (26) Inactivation titres with lightly coupled phage correlate to the presence of high affinity antibodies in the sera (5) The purpose of the present study was to compare the Pl A reactivities of rabbit sera after immunization with a hapten protein conjugate (NIP_1 CG see Methods) to the reactivities of the same sera in the HPI tests with standard and lightly coupled T-2 phages By this means we expected to determine the role of high affinity antibodies in the Pl A reaction

MATERIAL AND METHODS

Antigens The hapten used was 5 nitro-3 iodo-4 hydroxyphenylacetic acid (NIP) NIP, NIP azide and NIP ε aminocaproic acid were synthesized according to *Brownstone et al* (1) Conjugates of NIP and proteins (chicken globulin and bovine serum albumin) were also prepared as described by *Brownstone et al* (1)

Antisera Rabbits were immunized with one intraperitoneal injection of 2 mg alum precipitated $NIP_{12}CG$ (a preparation containing, on an average, 12 molecules of NIP per one molecule of chicken globulin) together with 10^{10} killed Haemophilus pertussis bacteria Blood samples were obtained 21 days later Sera were separated and stored at –20° C IgG fractions were prepared by DEAE cellulose chromatography at pH 7 5 as described by *Makela et al* (14) The purity of the IgG fractions was checked by cellulose acetate electrophoresis No contaminating IgM or IgA was detected

Antibody Determinations

Haptenated phage (T 2) inactivation (HPI) titres were determined by the method of *Makela* (13) HPI titres with lightly coupled phages were assayed according to *Koskimies et al* (5) Heavily coupled (standard) phage represented coupling with 0 16 per cent NIP azide and lightly coupled phage coupling with 0 005 per cent NIP azide

Antigen binding capacities were determined by the method of *Farr* (3) as modified by *Brownstone et al* (2) A high concentration of hapten ($N^{125}IP$ ε aminocaproic acid, 2×10^{-7} M) was used in order to estimate the total quantity of antibody present in the sera

Platelet aggregation (Pl A) test The micromethod described by *Penttinen and Myllyla* (21) and modified by *Penttinen et al* (25) was used A modified Ringer s solution (10) and individual lots of platelets (instead of pools) were used throughout the study, Only checkerboard titrations were made Sera were diluted with loops on disposable microplates (from 1 20 to 1 2560) in volumes of 0 025 ml/cup $NIP_{24}BSA$ (a preparation containing on an average 24 molecules of NIP per one molecule of bovine serum albumin) was used as antigen and was diluted from 1 1200 to 1 2 256 000 in tubes and pipetted then onto the plates (0 025 ml/cup) After 1 hr incubation at room temperature 0 05 ml of platelet suspension (200 000 platelets/ml) was added per cup The aggregation pattern of the platelets was observed after overnight incubation at 6-8° C Two or three parallel titrations of each serum were performed simultaneously with different lots of platelets In order to establish the medium sensitivity of platelets under the present test conditions

results from 167 successive checkerboard titrations of $NIP_{24}BSA$ antigen and our standard hyperimmune anti NIP serum (obtained by immunizing a rabbit with 3 subcutaneous injections of 1 mg alum precipitated NIP_1 CG, together with 10^{10} killed *Haemophilus pertussis* bacteria at 6–8 week intervals) were analyzed

Complement fixation (CF) tests were performed according to the standard micromethod of *Lennette* (6) by using two full units of complement (a stock of guinea pig serum) Antigen ($NIP_{24}BSA$) was diluted from 1 600 to 1 614 400 and sera from 1 20 to 1 2560 in checkerboard titrations The antigen was not anticomplementary at the dilutions used Most IgG fractions proved to be anticomplementary at dilutions 1 20–1 80

RESULTS

The sensitivity of platelets to aggregate upon stimulation with soluble antigen antibody complexes under the present test conditions was determined by analyzing results from checkerboard titrations of our standard NIP anti NIP complex with 167 individual lots of platelets The results are presented in Fig 1 Checkerboard titration patterns comprising a 'score' (number of positive antigen/antibody combinations per plate) of 31–50 were considered to represent medium sensitivity The 6 lots of platelets finally used for the study gave the following 'scores' 40 35 36 38 41 and 37 (mean 37 8) The mean of 152 successive 'scores' (from experiments in which the 'score' could be calculated) was 39 7 It is thus likely that the results from these series of tests were also well comparable with each other

The PLA and CF antibody response in the IgG fractions of 17 rabbits was studied by checkerboard titrations Anti NIP PLA titres ranged from < 20 to 320 and the PLA 'scores' from 0 to 36 Anti NIP CF titres were between < 20 and 640 CF 'scores' were not possible to calculate because of anticomplementarity of most of the fractions

The results of HPI titrations with standard and lightly coupled phage and determinations of antigen binding capacities of 10 IgG fractions together with PLA and CF reactivity data are presented in Table 1 In

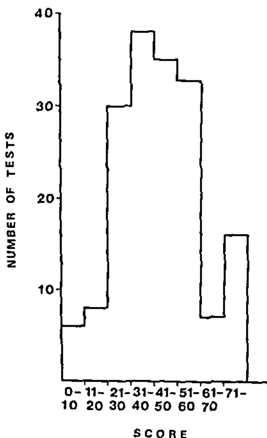


Fig 1 Distribution of 'scores' (number of positive antigen/antibody combinations per checkerboard titration) of a standard hyperimmune anti NIP serum and $NIP_{24}BSA$ antigen with 167 individual lots of platelets

these 10 preparations the antibody content was sufficient for reliable antigen binding capacity determinations In order to compare the reactivities of the IgG fractions of individual rabbits with each other the values of antibody activities were adjusted to represent equal antibody concentrations The modification of the Farr assay with a high concentration of antigen facilitates precipitation by also low affinity antibodies thus measuring the total amount of antibody (9 15) All values were adjusted to correspond to a binding of 2 00 mol/litre of fraction (calculated to represent a concentration of about 100 µg/ml of anti NIP IgG) These corrected values are presented in Table 2

TABLE 1 *Anti NIP Reactivities in Primary Response (3 Week) IgG Fractions of 10 Rabbits*

Rabbit No	Anti NIP titre with heavily coupled phage	Anti NIP titre with lightly coupled phage	Anti NIP Pl A titre	Pl A score *	Anti NIP CF titre	Anti complementarity (titre)	Antigen binding capacity†
2	277 500	25	80	29	40	<20	2 30
5	395 400	7	80	21	<80	80	1 35
8	4 878 000	64	160	28	160	80	14 95
9	3 663 000	116	160	26	ND	ND	44 95
10	411 400	12	40	18	80	<20	2 56
11	3 484 000	134	320	36	640	40	42 47
12	881 000	8	160	24	80	40	6 58
13	2 732 000	362	320	22	320	40	41 52
14	638 000	129	80	23	160	40	10 30
17	1 103 000	23	160	22	160	40	27 42

* number of positive antigen/antibody combinations in checkerboard titration
† binding of $N^{125}IP$ ε aminocaproic acid at a hapten concentration of 2×10^{-7} M Farr assay
ND — not done

The titres of heavily coupled (standard) HPI and the Pl A reactivities showed a positive correlation The correlation was most significant with the HPI titres and the Pl A 'scores' (the correlation coefficient for logarithmic values was 0 7943, corresponding to $p < 0\ 005$ by the Student s t test) The correlation of anti NIP Pl A titres to the HPI titres was less significant ($p < 0\ 05$) Anti NIP CF titres and Pl A titres were mostly of the same magnitude but their correlation was not statistically significant ($p < 0\ 2$) Most of the IgG fractions were anticomplementary, however no direct Pl A reactions (without added antigen) were registered There was no correlation of the Pl A reactivities to the titres of lightly haptenated phage inactivation which have been reported to correlate to the amount of very high affinity antibodies (5) The affinity threshold for lightly coupled HPI seems to be clearly higher than the affinity threshold for the Pl A technique The positive correlation of the standard HPI titres to the Pl A

TABLE 2 *Anti NIP Reactivities in Primary Response (3 Week) IgG Fractions of 10 Rabbits after Adjustment of Values to Equal Antibody Concentrations (200 Nanomoles of Hapten Bound/Litre of IgG Fraction Concentration of Free Hapten was Approximately 10^{-7} M)*

Rabbit No	Anti NIP titre with heavily coupled phage	Anti NIP titre with lightly coupled phage	Anti NIP PlA titre	Pl A score	Anti NIP CF titre
8	650 000	9	20	4	20
5	590 000	11	120	30	ND
10	320 000	9	30	18	60
12	270 000	2	50	10	25
2	240 000	22	70	25	35
11	170 000	6	15	0	30
9	160 000	5	10	0	ND
13	130 000	17	15	0	15
14	120 000	25	15	0	30
17	80 000	2	10	0	10

reactivities suggests that these two methods have similar requirements for primary response IgG affinity

DISCUSSION

The PlA method is a sensitive means for demonstrating immune complexes of small size antigens and their corresponding antibodies (23) There are however, certain disadvantages especially when a large number of specimens is to be tested The different sensitivities of individual lots of platelets and the requirement of daily preparations of platelets are the most obvious difficulties The score of NIP-BSA antigen and hyperimmune anti NIP serum checkerboard titrations (number of positive antigen/antibody combinations per test) has been found to be a good indicator for the platelet sensitivity (25) In this study the effect of different sensitivities of various lots of platelets were avoided by using only lots whose scores were very close to the mean of several successive titrations By this means comparable results with different lots of platelets were obtained

Determination of scores from checkerboard titrations may be a more informative index of PlA reactivity than the optimal antibody titres alone at least with antiviral antibodies (17) In the present study of anti NIP IgG fractions the standard HPI titres correlated to the PlA scores more significantly than to the anti NIP PlA titres The quantitative threshold for detection of 3 week primary response IgG by the PlA technique could not be expressed accurately on the basis of the present experiments since quantitative precipitations were not performed A relatively rough estimate of antibody content based on HPI titres with heavily coupled phage can however be calculated according to *Sarvas and Makela* (26) A quantity of 1 μg/ml of primary response anti NIP IgG was reported to correspond to a concentration of approximately 2100 phage inactivation units/ml The threshold amount of anti NIP IgG detectable with the PlA method would then be of the order of 1 7–14 6 μg/ml in the 10 IgG fraction presented It has been shown previously that 0 3 μg/ml of hyperimmune anti NIP antibody can be detected with the PlA technique (23) Thus about 5–50 times higher concentrations of primary response than hyperimmune anti NIP is probably needed to cause PlA in the presence of the appropriate antigen

Direct PlA reactions (without the addition of antigen) did not correlate to anticomplementarity in the rabbit IgG fractions although aggregated IgG is most likely responsible for both of these phenomena When human gammaglobulin was aggregated by various chemicals the platelet activating (as estimated by release of ^{14}C serotonin) and CF abilities of the aggregated products were usually very similar (7) Rabbit IgG may be have differently in this respect

Studies of anti NIP reactivities of the IgG fractions did not reveal any great differences between CF and PlA methods The minimum amount of CF reactive anti NIP IgG detectable (according to the calculation procedure used above) would range between 1 8–14 6 μg/ml PlA reactivities of antiviral antibodies probably develop somewhat later than CF reactivities (17–19) Three weeks after immunization (when definite maturation of immune response has already taken place) approximately similar antibody titres were seen with both CF and PlA methods although the titres were not statistically significantly correlated

Fauci et al (4) showed that the efficiency of CF was related to the affinity of anti DNP IgG in pooled rabbit sera taken 25 days after immunization with a single dose of antigen They found that high affinity IgG (prepared by immunoadsorbent technique), with an intrinsic association constant (K_0) 900 $\times$ 10^6 litres/mol could be detected in an amount of 2 1 μg/ml A preparation of medium affinity IgG (K_0 10 $\times$ 10^6 litres/mol) was however, detected as effectively The minimum amount of low affinity IgG (K_0 1 1 $\times$ 10^6 litres/mol) de

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tected was 8 3 μg/ml These results are in accordance with our hypothesis that very high-affinity antibody is not critical to either Pl A or CF measurements of primary response anti NIP IgG It remains to be determined whether the CF reactivity of IgG in earlier stages of an immune response (1–2 weeks) is less dependent on affinity than the Pl A reacticity It is also possible that IgG subclasses not fixing complement exist in rabbits and are involved in the mechanism of the Pl A reaction

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SENSITIZED SHEEP RED CELLS AS A REACTANT FOR *STAPHYLOCOCCUS AUREUS* PROTEIN A

Methodology and Epidemiology with Special Reference to Weakly Reacting Methicillin-Resistant Strains

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Staphylococci with cell bound protein A reacted with sensitized sheep red cells in a simple slide reaction with agglutination Extracellular protein A proved capable of preventing the sensitized sheep red cells from reacting with cultures of *Staph aureus* strains containing protein A This confirms the reactivity of protein A with free Fc fragments of IgG on the surface of the sensitized sheep red cells as the cause of haemagglutination The choice of culture medium proved important for demonstrating protein A Peptone free media proved clearly unsuitable 341 routinely isolated strains of *Staph aureus* were examined for agglutination of sensitized sheep red cells in the slide test and for tube agglutination of sensitized sheep red cells with broth culture filtrate 88 3 per cent of the strains were positive if examined on slides, while 6 7 per cent of the strains contained no cell bound or free protein A demonstrable by either method Among the methicillin resistant strains 50 per cent showed no agglutination on slide Antibiotic sensitive strains belonging to phage group II tended to have weaker protein A reactivity than other groups.

After *Jensen's* (9 10) demonstration of an antigen specific for *Staph aureus,* called antigen A investigation by *Löfkvist* & *Sjöquist* (16 17) have shown that this antigen is a protein and that it reacts with serum globulins *inter alia* from man and rabbit

The reaction of the serum with protein A was first conceived as an antigen-antibody reaction but *Forsgren* & *Sjoquist* (5) showed that it is a pseudo-immune reaction in which protein A reacts with the Fc part and not with the Fab parts of the IgG molecule These observation have since been confirmed by the use of different methods in investigations by *Forsgren* (6) *Forsgren et al* (7), *Kronvall* & *Williams* (11), *Kronvall* & *Frommel* (12) and by *Lind et al* (14)

The occurrence of protein A in staphylococcal strains has been demonstrated by various methods Precipitation of extract from bacterial cultures with serum (*Jensen* 9, 10 *Löfkvist* 18, *Kronvall* & *Williams* (11), *Kronvall* & *Frommel* (12) may be regarded as the fundamental method Absorption of radioactive myeloma globulins of IgG type to staphylococci was used by *Kronvall et al* (13), *Lind* (15) used non specific fluorescence as a sign of binding of serum globulin *Forsgren* (6) demonstrated agglutination of sensitized sheep red cells by supernatant from broth culture of *Staph aureus* in test tubes All the investigators found that about 90 per

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cent of the *Staph. aureus* strains showed protein A reactivity and *Forsgren* (6) reported that occasional coagulase-negative staphylococci also might present such reactivity.

The important observation by *Forsgren & Sjoquist* (5) that the Fc part of IgG reacts with protein A prompted us to use sensitized sheep red cells as a relatively simple system for demonstrating such reactivity. This is analogous to investigations for rheumatoid factor which also reacts with the Fc part of the IgG molecule (*Henney & Ishizaka* 8; *Winblad et al.* 24).

MATERIAL AND METHODS

Sheep erythrocytes. Sheep blood was collected in standard ACD adenine solution. The red blood cells were washed three times in saline and resuspended to a 25 per cent saline suspension. After haemolysis with distilled water the strength of the red blood cell suspension was checked with a Linson photometer.

Rabbit anti sheep erythrocyte serum. Rabbits were immunized by 2 ml 25 per cent sheep red cells applied intravenously twice weekly during five weeks to give an agglutination titre of at least 1/5000. Collected serum was diluted 1/2 in sterile glycerol solution and was kept at 4°C.

Sensitized sheep erythrocytes. One fourth of the minimum agglutinating dose was used as sensitizing dose. This dose in 0.1 ml serum was used for 100 ml of 2 per cent sheep red cells. The preparation of sensitized sheep erythrocytes was the same as that used for the detection of rheumatoid serum factor as described by *Anderson et al.* (1). The sheep cells were washed with saline after sensitization.

Staphylococcus aureus strains. Strain Cowan I and Wood 46 were chosen as protein A positive and negative controls respectively. 341 strains of *Staph. aureus* recently isolated from routine diagnostic material were studied. To some extent the material was selected for multiple antibiotic resistance. All strains of *Staph. aureus* were coagulase-positive and were also classified with regard to phage type and antibiogram. Reisolate from the same patient was avoided. In a special study of methicillin resistant strains the material consisted partly of strains from Malmo, partly of a collection of strains received from Dr. *Forsgren*, Uppsala and derived from other Swedish and international sources: 34 strains originating from Lund (4), Uppsala (11), Örebro (5), Australia (2), Boston (4), Hungaria (3), Liverpool (1), Zurich (4). A number of *Staph. epidermidis* strains from human sources have been studied.

Media

Human blood agar containing peptone was prepared as described by *Nilého & Sjostrōm* (20). Outdated blood bank material was used. After removal of plasma the packed red cells were resuspended in meat extract broth before addition to the medium. The medium contained 0.2 per cent proteose peptone No. 3 (Difco).

Human blood agar without peptone was prepared as above excluding proteose peptone No. 3.

0.3 CYT agar was prepared according to *Novick & Roth* (21).

Haematine agar with IsoVital X. Double layered plates were used.

Lower layer: Beef extract (Difco) 0.5 per cent, sodium chloride 0.3 per cent, secondary sodium phosphate (Merck) 0.2 per cent, Japanese shred agar 1.4 per cent, distilled water ad 100 per cent.

Upper layer: Beef extract (Difco) 0.5 per cent, proteose peptone No. 3 (Difco) 1.0 per cent, sodium chloride 0.3 per cent, secondary sodium phosphate (Merck) 0.2 per cent, Japanese shred agar 1.1 per cent, distilled water ad 100 per cent.

60 per cent suspension of plasma free erytrocytes in meat extract broth, prewarmed at 80°C for twenty minutes, was then added to a concentration of 8 per cent. Before pouring the plates, IsoVital X (BBL) was added to a concentration of 0.25 per cent.

Chapman agar. Bacto Mannitol Salt Agar (Difco).

Tween 80 agar was prepared according to *Sierra* (23).

Water Blue agar. Bacto-peptone (Difco) 1.2 per cent, NaCl 0.3 per cent, $Na_2HPO_4 \times 2H_2O$ 0.2 per cent, glucose 0.2 per cent, Bacto-agar (Difco) 1.8 per cent, Tween 80 (Atlas Powder) 0.01 per cent, diacetin (BDH) 0.05 per cent, Wasserblau (Merck, Darmstadt) 0.01 per cent (modified according to *Ericson* 3).

Slide agglutination test for protein A on bacteria. A small amount of staphylococcal culture from a solid medium was mixed with one drop of 3 per cent sensitized sheep red cells with the aid of a platinum loop on a microscopic slide. The mixture was examined for up to 5 minutes during continued tilting of the glass to and fro. Positive results were observed in the form of a strong, rapidly developing haemagglutination. As a positive control we used the high protein A strain, Cowan I, and as a negative control the protein A negative strain Wood 46, as well as non-sensitized sheep red cells together with Cowan I and the strain to be tested.

Tube agglutination test for bacterial protein A. A small amount of *Staph. aureus* culture on solid medium was mixed with 0.1 ml NaCl in a test tube, to which 0.1 ml 1 per cent sensitized sheep red cells was added. The tube was incubated for two hours at 37°C, after which the results were read.

It was then incubated for a further 16–18 hours at +4°C after which it was shaken again and the results were re read

Tube agglutination test for soluble protein A in broth supernatant or filtrate To a test tube containing 0 2 ml supernatant of a centrifuged 18 hours broth culture in meat extract broth, incubated with agitation at 37°C or with an equal amount of the supernatant after filtration through Millipore filter APD 0 22 mμ was added 0 25 ml washed 1 per cent sensitized sheep red cells The tube was checked for agglutination after incubation as described above

In the tube agglutination test severe haemolysis sometimes made it difficult or impossible to read the result, especially if the culture had been incubated over night

Phage typing The technique described by *Blair & Williams* (2) was used with the standard phage set

Sensitivity tests The disc diffusion method described by *Ericsson* (4) was used for antibiotic sensitivity tests The following antibacterial agents were used sulphonamide benzyl penicillin methicillin cephalosporin gentamicin kanamycin, streptomycin erythromycin lincomycin tetracycline fusidic acid and chloramphenicol

RESULTS

Slide Agglutination of 3 Per Cent Sensitized Sheep Red Cells by Staphylococci

Cultures of strain Cowan I showed strong reactivity in the slide test, that is a clear agglutination within a few seconds Such reactivity was also demonstrated for most of other strains of *Staph aureus* Strain Wood 46 showed no agglutination on slide, neither did some ten strains of *Staph epidermidis* A few strains of *Staph aureus* did not either show any clear agglutination on slides No agglutination was shown by Cowan I or other *Staph aureus* strains if non sensitized sheep red cells were used Variation of the concentration of the suspension of sheep red cells within the range of 1–5 per cent proved to have little or no effect on the strength of the reactions A 3 per cent suspension of sensitized sheep red cells was most suitable and selected for routine use

Tube Agglutination with Bacterial Cells

When the culture had proved positive with the slide method tube agglutination was generally also observed within 2 hours at 37°C and always within 24 hours at 37°C Haemolysis of the culture sometimes made it difficult to read the reaction if incubated for 24 hours

Tube Agglutination with Culture Filtrate

Such agglutination was closely correlated with agglutination by bacterial cells In a few cases, however, it was positive without any demonstrable agglutination on slides by bacterial cells In such cases it was assumed that only extracellular protein A was present

Extract of protein A from the strain Cowan I prepared according to *Jensen* (9)* showed no agglutination in the slide or tube method

Inhibition of Agglutination

Filtrates from broth cultures of *Staph aureus* containing protein A mixed with sensitized sheep red cells on microscopic slides sometimes inhibited agglutination by subsequent addition of Cowan I culture This phenomenon did not appear if non inoculated broth was used or cultures were known to be protein A negative using other methods In the same way protein A on strain Cowan I was apparently blocked if culture of this strain had been incubated with rabbit or human serum before the sensitized red sheep cells were added

Dependence on Reaction of Culture Medium

Protein A positive strains were cultured on different media and tested for reactivity with sensitized sheep red cells on slides It was found that the use of medium without peptone did not result in detectable protein A synthesis A medium such as haematin agar with IsoVital X promoted production of protein A The results obtained with other media varied 0 3 CYT agar according to *Novick & Roth* (21) gave fairly good results and was used for the epidemiological investigations described below

* Kindly supplied by Dr *Kronvall*

TABLE 1 *Demonstration of* Staph aureus *Protein A Using Sensitized Sheep Red Cells*

Positive in slide test	301 (88 3 %)
Negative on slide but positive in tube test with *bacterial cells*	11 (3 2 %)
Positive only with culture filtrate in tube agglutination test	6 (1 8 %)
No demonstrable protein A	23 (6 7 %)
Total	341 (100 %)

Protein A Content of 341 Strains of Staph aureus

It appears clearly from Table 1 that 88 3 per cent of the strains showed a positive slide agglutination and 3 2 per cent negative slide agglutination but positive tube agglutination with bacterial cells 1 8 per cent showed reaction only with extracellular protein A 6 7 per cent showed no demonstrable protein A with the method used

The material is divided according to the antibiogram and phage type (Table 2) Positive slide agglutination was less common among the methicillin resistant strains in the multiple resistant group and also among strains belonging to phage group *II* in the more susceptible group This applies in particular to methicillin resistant strains in half of which no protein A at all could be demonstrated with the methods used

Extended Examination of Methicillin Resistant Strains

Since the results of the examination of methicillin resistant strains isolated in Malmo appeared remarkable methicillin resistant strains of other origins were also examined Table 3 gives the results of this investigation of 22 freshly isolated strains from Malmo and 34 strains from other places It was found by both methods that half of the Malmo strains were negative, while strains collected from other places showed a tendency to protein A reactivity only of the extracellular type

DISCUSSION

Methodological Consideration

From a methodological point of view distinction may be made between a) cell bound b) extracellular and c) extracted protein A Cell bound protein A should then be understood as such protein A as can be demonstrated by agglutination of sensitized red

TABLE 2 *Distribution of* Staph aureus *Strains According to Protein A Reactivity*

	Sensitive or resistant to at most two antibiotics			Multiresistant		
	Phage group I	Phage group II	Others	Methicillin resistant	Phage group 80/81	Others
…tive in sl de-test	54 (98 1 %)	28 (71 8 %)	63 (94 0 %)	9 (42 9 %)	69 (92 0 %)	78 (92 8 %)
…gative on slide but positive in tube with bacterial cells		3 (7 7 %)		2 (9 5 %)	4 (5 3 %)	2 (2 4 %)
…tive only with culture filtrate in tube agglutination test		3 (7 7 %)			1 (1 3 %)	2 (2 4 %)
…o demonstrable protein A	1 (1 8 %)	5 (12 8 %)	4 (6 0 %)	10 (47 6 %)	1 (1 3 %)	2 (2 4 %)
…tal	55	39	67	21	75	84

It was then incubated for a further 16-18 hours at +4°C after which it was shaken again and the results were re-read.

Tube agglutination test for soluble protein A in broth supernatant or filtrate. To a test tube containing 0.2 ml supernatant of a centrifuged 18 hours broth culture in meat extract broth inoculated with agitation at 37°C or with an equal amount of the supernatant after filtration through Millipore filter APD 0.22 mμ was added 0.25 ml washed 1 per cent sensitized sheep red cells. The tube was checked for agglutination after incubation as described above.

In the tube agglutination test severe haemolysis sometimes made it difficult or impossible to read the result, especially if the culture had been incubated over night.

Phage typing. The technique described by *Blair & Williams* (2) was used with the standard phage set.

Sensitivity tests. The disc diffusion method described by *Ericsson* (4) was used for antibiotic sensitivity tests. The following antibacterial agents were used: sulphonamide, benzyl penicillin, methicillin, cephalosporin, gentamicin, kanamycin, streptomycin, erythromycin, lincomycin, tetracycline, fusidic acid and chloramphenicol.

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Cultures of strain Cowan I showed strong reactivity in the slide test, that is a clear agglutination within a few seconds. Such reactivity was also demonstrated for most of other strains of *Staph. aureus*. Strain Wood 46 showed no agglutination on slide, neither did some ten strains of *Staph. epidermidis*. A few strains of *Staph. aureus* did not either show any clear agglutination on slides. No agglutination was shown by Cowan I or other *Staph. aureus* strains if non-sensitized sheep red cells were used. Variation of the concentration of the suspension of sheep red cells within the range of 1–5 per cent proved to have little or no effect on the strength of the reactions. A 3 per cent suspension of sensitized sheep red cells was most suitable and selected for routine use.

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When the culture had proved positive with the slide method, the agglutination was generally also observed within 2 hours at 37°C and always within 24 hours at 37°C. Haemolysis of the culture sometimes made it difficult to read the reaction if incubated for 24 hours.

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Extract of protein A from the strain Cowan I prepared according to *Jensen* (9)* showed no agglutination in the slide or tube method.

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Filtrates from broth cultures of *Staph. aureus* containing protein A mixed with sensitized sheep red cells on microscopic slides sometimes inhibited agglutination by subsequent addition of Cowan I culture. This phenomenon did not appear if non-inoculated broth was used or cultures were known to be protein A negative using other methods. In the same way protein A on strain Cowan I was apparently blocked if culture of this strain had been incubated with rabbit or human serum before the sensitized red sheep cells were added.

Dependence on Reaction of Culture Medium

Protein A positive strains were cultured on different media and tested for reactivity with sensitized sheep red cells on slides. It was found that the use of medium without peptone did not result in detectable protein A synthesis. A medium such as haematin agar with IsoVital X promoted production of protein A. The results obtained with other media varied. 0.3 CYT agar according to *Novick & Roth* (21) gave fairly good results and was used for the epidemiological investigations described below.

* Kindly supplied by Dr. Kronvall.

strains isolated in Denmark. On the other hand, *Forsgren* (6) did not find methicillin-induced resistance to result in loss of protein A production.

Whether the tendency of methicillin resistant strains to react only weakly or to be negative has any biological connection with the property of methicillin-resistance is debatable.

The methicillin resistant strains isolated in Malmö generally showed a weaker protein A reaction than strains isolated elsewhere in Sweden or abroad. The latter included no strains from Denmark. Since nearly all Malmö-strains belonged to the same epidemic (with one type imported from Denmark), while other strains belonged to different epidemics with relatively different phage types, it is clear that it is not possible on the basis of frequency studies to discuss any relationship between protein A-reactivity and other properties characteristic of the Malmö-strains.

The question is whether similar epidemiological situations may be responsible for the tendency to weak protein A-reactions among strains of phage group II, which were found in the present material. A large number of group II strains in the present material originated from cases of impetigo, which might be of interest (*Parker* 22). No epidemiological relationship between the different strains in group II is known.

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THE OCCURRENCE OF MEASLES ANTIGEN PRECIPITATING ANTIBODIES IN SERA FROM PATIENTS WITH MULTIPLE SCLEROSIS AND THEIR RELATIVES

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The gel precipitation reaction between measles antigen and sera from multiple sclerosis (MS) patients their children, siblings and parents compared to those of controls from families without known cases of MS were studied The frequency of positive gel precipitation (GP) reaction is higher among MS patients than among controls. The siblings of MS patients have a higher per cent of positive GP reaction than the controls but smaller than MS patients The frequency of positive GP reaction decreases with increasing age Women have a higher per cent of positive GP reaction of measles antibody than men The positive correlation between hemagglutination inhibition (HI) and GP reaction was shown Only IgG was immunologically active against measles antigen

In recent years, a new group of diseases involving the central nervous system has been described These diseases, being of viral aetiology, are characterized by a long incubation period and a relatively slow progressive course They are called "slow virus diseases" (12)

Research in recent years deals with the possibility that MS is a slow virus disease of the central nervous system such as Kuru and Creutzfeldt s Jakob diseases (3, 7, 11, 12, 13) Measles virus is presumed to be the aetiological agent in subacute sclerosing panencephalitis (SSPE) The aetiology of this disease was confirmed by isolation of infectious measles virus from the brain of SSPE patients (15)

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High measles antibody titres in serum and cerebrospinal fluid of these patients provide further evidence (5, 22)

There is growing evidence to suggest a relationship between the measles virus or another related myxovirus and MS Higher titres of measles antibody have been found in the serum of the MS patients as compared to the control subjects (1, 2, 14, 23, 24)

The methods employed for measuring measles antibodies have varied from complement fixation, serum neutralization of measles virus hemagglutination inhibition reactions, immune adherence, platelet aggregation, immunofluorescent methods and gel precipitation tests Many of these methods have, however, only been applied to relatively small MS material

Since we have the opportunity to contact most MS patients in the Copenhagen area

(1.5 million people) the present report describes the frequency of the gel precipitation reaction between the measles antigen and sera from these MS patients, their children, siblings and parents. The data are compared to those obtained from families without known cases of MS.

MATERIAL AND METHODS

Series of Patients

The individuals studied were 128 patients (74 females), 102 siblings of MS (56 females), 83 children of MS (41 females) and 30 parents of MS (18 females). Diagnosis was made using the clinical criteria of Broman et al. (6) and McAlpine et al. (18).

Controls

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Gel Precipitation Antigens

3 ml of undiluted virus suspension possessing cytopatic effects in dilution up to 10^{-6} and 80 ml of medium 199 were introduced into 6 day-old cultures. Cultures were incubated at 37° C without changing the medium until complete degeneration of the cells had occurred (5 to 7 days after virus inoculation). The cells were mechanically detatched and the whole culture sonificated for 15 min at 50 Watt using a Branson Sonifier B 12. The cell debris was removed by low speed centrifugation (1000 g, 10 min at room temperature) and then the supernatant was concentrated twenty times by means of lyofilization. Antigen was stored at −20° C and sonificated again immediately before use. The procedure for preparing control antigen was identical except that the cells were not inoculated with measles virus.

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Gel precipitation tests were carried out on the microscope slides by the double immunodiffusion method (20, 21). The wells (15 µl volume) were situated circularly around a central antigen well with the holes about 5 mm from each other. Agarose (L'Industrie Biologique Francaise S.A.) was used in a concentration of 1 per cent (w/v) in 0.9 per cent (w/v) aqueous NaCl. The gel also contained 0.01 per cent sodium azide. Matrix holes were filled with 15 µl of reactants. Hereafter the slides were placed in water saturated immunodiffusion chambers. The precipitations were allowed to form at room temperature for 48 hours. After immunodiffusion was complete, the slides were washed free of unreacted antigen and antibody in saline for two days. After washing, saline was washed out in distilled water for about three hours (8). After drying the slides were stained in Amido Black (28). Excess of stain was washed away with 2 per cent acetic acid. The presence or absence of precipitation lines was read from the slides.

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Control of Specificity of the Gel Precipitation Reaction

Isolation of serum IgG. 4 ml MS serum diluted three times with tap water was precipitated at 2.0 M ammonium sulphate (pH 6.8). The precipitate was dissolved in 6 ml 0.005 M phosphate buffer (pH 8.0) and dialysed against 1000 ml of this phosphate buffer. Final purification of the

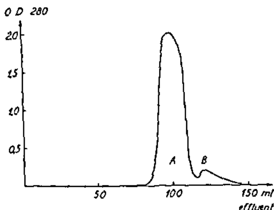


Fig 1 DEAE cellulose chromatography of the MS serum protein fraction precipitating at 2 0 M ammonium sulphate (pH 6 8) Initial buffer 0 005 M phosphate (pH 8 0) Final buffer 0 3 M phosphate (pH 8 0) (For experimental details cf the text)

IgG present in the 2 0 M ammonium sulphate precipitate was achieved by chromatography on DEAE-cellulose columns (10 26) 4 ml of the dialysate was applied on the column 22×3 cm (internal diameter) Elution was performed by increasing the concentration of the phosphate buffer (pH 8 0) continuously (0 005 M to 0 3 M) by a double siphon stand containing these two buffers The flow rate was 30 ml per hour and 5 ml fractions were collected on a LKB Ultrarac automatic fraction collector (LKB Stockholm Sweden) The protein extinction of each 5 ml fraction was measured at 280 nm in a Beckman Spectrophotometer (Fig 1) The two absorption peaks corresponded to protein fraction A and B The eluates containing protein fraction A and fraction B were concentrated to about 1 g protein per 100 ml by vacuum dialysis Immunoelectrophoresis of fraction A and B (using antiserum against human serum proteins from the goat) revealed that both fractions contained exclusively IgG of intermediate electrophoretic γ mobility (Fig 2) The protein fractions A and B were collected in order to prove which serum proteins were able to precipitate measles antigen from infected Vero cells

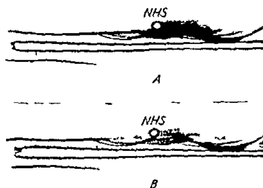


Fig 2 Micro immunoelectrophoresis of purified IgG developed with goat antiserum against normal human serum

Immunoelectrophoresis of the Fractions A and B eluted with phosphate buffer during DEAE cellulose chromatography of the serum protein fraction precipitating at 2 0 M $(NH_4)_2SO_4$ (pH 6 8) (For experimental details cf the text)

Center trough 80 µl goat antiserum against human serum

Upper hole 1 5 µl (1 per cent w/v) normal human serum (NHS)

Lower hole 1 5 µl (1 per cent w/v) of either Fraction A or B

RESULTS

Assay of Immunochemical Specificity

Initial studies revealed only IgG isolated by salting out and column chromatography to precipitate measles antigen prepared from measles infected Vero cells Thus, as shown in Fig 3, no serum proteins precipitating with ammonium sulphate above 2 0 M (pH 6 8) cause precipitation of the measles antigen Thus among these proteins, serum albumin does not make artificial lines (cf the survey by *Clausen* (8)) Comparison of Figs 2 and 3 reveals, on the other hand that purified IgG with intermediate electrophoretic mobility precipitates the measles antigen

Assay of Composition and Activity of Measles Antigen from Measles Infected Vero Cells (MA)

Measles antigen was characterized by two values i e protein and ribonucleic acid (RNA) content

The protein concentration was 13 0 mg per ml The RNA value expressed as µg of ribose per ml measles antigen was 28 6, which corresponds to 2 2 µg ribose per mg protein Therefore, the amount of MA used in GP tests (15 µl) corresponded to 195 µg or to 429 ng protein or ribose, respectively

(1.5 million people) the present report describes the frequency of the gel precipitation reaction between the measles antigen and sera from these MS patients, their children, siblings and parents. The data are compared to those obtained from families without known cases of MS.

MATERIAL AND METHODS

Series of Patients

The individuals studied were 128 patients (74 females), 102 siblings of MS (56 females), 83 children of MS (41 females) and 30 parents of MS (18 females). Diagnosis was made using the clinical criteria of *Broman et al.* (6) and *McAlpine et al.* (18).

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TABLE 1 *Gel Precipitation Reaction of Sera from Patients with MS, Their Children, Siblings, Parents and from Controls, against Measles Antigen Prepared from Measles Infected Vero Cells*

Group of sera		Mean age years	Negative number	Positive number	Positive % of total
MS female		48	31	43	58
MS male		49	28	26	48
Daughters		27	16	25	60
Sons		22	19	23	54
Sisters		45	32	24	42
Brothers		50	31	15	32
Mothers		66	12	6	33
Fathers		69	12	0	0
Controls female	< 30 years	22	8	9	52
Controls female	≥ 30 years	46	20	8	28
Controls male	< 30 years	22	6	7	53
Controls male	≥ 30 years	49	14	2	12

positive reaction as the MS females (58 per cent) Sisters and mothers of MS patients have a lower frequency, 42 per cent and 33 per cent respectively The lowest frequency of precipitation formation was observed in the groups of female controls older than 30 years (28 per cent) Female controls younger than 30 years have *52 per cent positive reaction* In the groups of females mentioned the differences between MS patients and controls older than 30 years are significant ($p < 0.02$) and the same applies to daughters and controls older than 30 years ($p < 0.02$), as measured by the chi-square test

Among males the highest per cent of MA positive reaction was found among the sons of MS patients (54 per cent), this figure is of a size almost equal to that found in the controls younger than 30 years (53 per cent)

MS males have a higher frequency of antibody (48 per cent) than brothers of MS patients (32 per cent) A lower percentage of measles antibody positive reactions was found *in the group of controls older than 30 years* (12 per cent), it was lowest in fathers of MS patients (0 per cent)

TABLE 2 *The Levels of Significance of the Differences in Frequency of Positive Gel Precipitation Reaction with a Measles Antigen of Measles Infected Vero Cells Precipitating Sera from MS Patients Compared to the Frequency of this Reaction with Sera of other Groups The Evaluation was made by the Chi-Square Test*

Group of sera	Comparison with MS patients
Children of MS	NS
Siblings of MS	$p < 0.02$
Parents of MS	$p < 0.001$
Controls < 30	NS
Controls ≥ 30	$p < 0.001$

NS = non significant

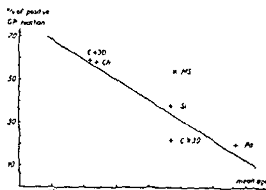


Fig 4 Plot of the frequency of GP positive reaction versus mean age The regression line for the groups *without MS has a slope of* -0.93 ± 0.15

MS MS patients

Ch Children of MS

Si Siblings of MS

Pa Parents of MS

C < 30 Controls < 30 years

C ≥ 30 Controls ≥ 30 years

In the male groups the difference between MS patients and controls older than 30 years is significant ($p < 0.01$), and the same applies to MS patients and their fathers ($p < 0.01$) to sons and fathers ($p < 0.001$), to brothers and fathers of MS patients ($p < 0.01$) to sons of MS patients and to control males older than 30 years ($p < 0.01$) as calculated by the Fisher test

Without correction for sex difference (Table 2) the frequency of positive precipitation reaction with serum from MS patients, compared to their siblings parents and controls older than 30 years is significant $p < 0.02$ $p < 0.001$, $p < 0.001$, respectively as measured by the chi square test The difference between siblings of MS patients and controls older than 30 years is non significant ($p < 0.1$) There is no significant difference between children of MS patients and matched controls younger than 30 years

Fig 4 shows a plot of the frequency of positive GP reaction versus the mean age for the groups mentioned in this paper Regression analysis of data from the five groups without MS reveals a regression line with the slope -0.93 ± 0.15 which is significantly different from 0 ($p < 0.005$), showing that occurrence of measles antibody decreases with increasing mean age

The relation of GP Reaction of HI Titres in the MS Patients and the Control Sera

The per cent of GP reaction as visualized by the Ouchterlony immunodiffusion method using our own measles antigen and the HI titres made with the commercially available measles antigen (treated with Tween and ether vide supra) are indicated in Tables 3 and 4 Among the sera with low titres there were only a few forming precipitation lines to MA With increased HI titres the per cent of positive immunodiffusion reaction increases in MS and control sera Almost all sera with titres higher than 128 exhibited precipitation lines in the gel test

The difference between per cent of GP positive sera in two groups of the MS patients i e patients with HI titres lower than 128 and those with HI titres ≥ 128 is significant on the level $p < 0.001$ calculated by the chi square test The same rule is valid also for control sera and the difference is also significant ($p < 0.001$)

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HI titre	GP negative sera	GP positive sera	Per cent of GP positive
≤8	14	1	7
16	11	2	13
32	10	2	14
64	10	6	37
128	7	8	53
256	1	9	90
≥512	0	6	100

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HI titre	GP negative sera	GP positive sera	Per cent of GP positive
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32	16	2	11
64	11	9	45
128	4	7	63
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DISCUSSION

Our initial studies revealed that the measles antigen prepared does precipitate in the GP test with the IgG fraction of human serum Thus the GP test reflects a specific immunochemical reaction and not a false reaction due for instance to complex formation with albumin

Studies by *Panelius et al* (23) have shown differences a higher frequency of measles antibody in MS than in controls as shown by gel precipitation method The present studies were undertaken in order to evaluate

this difference in a greater material, and in order to compare it to our previous results obtained by the HI test The serum of MS patients in this series has a significantly higher per cent of positive GP reaction than that observed in a control group in which the age distribution was the same This is in agreement with data reported by *Panelius et al* (23) However, our number of positive results is lower (controls ≥ 30 years 23 per cent, MS 54 per cent) than that found by *Panelius* (controls 76 per cent, MS 94 per cent) The percentage disagreement could be due to the different methods employed in the two studies The gel precipitation tests made by *Panelius et al* (23) were carried out on 5×5 cm glass photographic slides with a microtechnique described by *Wadsworth* (27) and modified by *Krause and Raunio* (16) It could also be explained on the basis of ethnic differences Recently, we have had the opportunity of comparing the measles antigen used by *Panelius et al* (23) to our own We found quite similar protein and RNA contents (14 mg protein and 32 µg ribose per ml), and the GP test gave an identical number of lines, maximum three in our system, not four or five as found by *Panelius* These data thus favour the last mentioned hypothesis.

The present paper also deals with relatives of MS patients The siblings of MS patients have a higher frequency of positive GP reaction than the controls but lower than that of MS patients, confirming the data of *Ammitzboll and Clausen* (2), *Brody et al* (4), *Henson et al* (14) and *Panelius et al* (24)

Comparison of different age groups reveals that the frequency of positive GP reaction decreases with increasing mean age

The present communication also demonstrated a correlation between sex and measles antibody, showing that women have a higher per cent of positive GP reaction than men This is in agreement with results obtained by the HI test (2)

The present data show a positive correlation between HI titres and GP reaction The same correlation was revealed by *Salmi* (25) for rubella antigen However, no correlation was previously found with the measles antigen used by *Panelius et al* (23)

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Our initial studies revealed that the measles antigen prepared does precipitate in the GP test with the IgG fraction of human serum Thus the GP test reflects a specific immunochemical reaction and not a false reaction due for instance to complex formation with albumin

Studies by *Panelius et al* (23) have shown differences a higher frequency of measles antibody in MS than in controls as shown by gel precipitation method The present studies were undertaken in order *to evaluate*

this difference in a greater material, and in order to compare it to our previous results obtained by the HI test The serum of MS patients in this series has a significantly higher per cent of positive GP reaction than that observed in a control group in which the age distribution was the same This is in agreement with data reported by *Panelius et al* (23) However, our number of positive results is lower (controls ≥ 30 years 23 per cent MS 54 per cent) than that found by *Panelius* (controls 76 per cent, MS 94 per cent) The percentage disagreement could be due to the different methods employed in the two studies The gel precipitation tests made by *Panelius et al* (23) were carried out on 5×5 cm glass photographic slides with a microtechnique described by *Wadsworth* (27) and modified by *Krause and Raunio* (16) It could also be explained on the basis of ethnic differences Recently, we have had the opportunity of comparing the measles antigen used by *Panelius et al* (23) to our own We found quite similar protein and RNA contents (14 mg protein and 32 μg ribose per ml) and the GP test gave an identical number of lines, maximum three in our system not four or five as found by *Panelius* These data thus favour the last mentioned hypothesis

The present paper also deals with relatives of MS patients The siblings of MS patients have a higher frequency of positive GP reaction than the controls but lower than that of MS patients confirming the data of *Ammitzboll and Clausen* (2), *Brody et al* (4), *Henson et al* (14) and *Panelius et al* (24)

Comparison of different age groups reveals that the frequency of positive GP reaction decreases with increasing mean age

The present communication also demonstrated a correlation between sex and measles antibody, showing that women have a higher per cent of positive GP reaction than men This is in agreement with results obtained by the HI test (2)

The present data show a positive correlation between HI titres and GP reaction The same correlation was revealed by *Salmi* (25) for rubella antigen However, no correlation was previously found with the measles antigen used by *Panelius et al* (23)

The aetiology of MS cannot be clearly explained on the basis of the present studies, the findings are compatible, however, with the concept that the host responce to measles, or to a closely related virus is due to a genetically controlled immunological defect in the MS families, since the frequency of positive GP reaction is higher among siblings to MS patients than among controls

These studies have been supported by a grant from *Ferdinand and Ellen Hindsgaul's fund* Copenhagen Denmark which supplied the equipment used and the *Danish Multiple Sclerosis Society* The authors are indebted to Miss *Susanne Blomsterberg* for her assistance in the hemagglutination inhibition test and to Mr *Axel Stoffregen* for the invaluable help in contacting the MS patients

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ON THE MECHANISM OF THE RING ZONE EFFECT OBTAINED WITH THE MIXED HAEMADSORPTION TECHNIQUE

Studies with Defined Model Systems

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Studies on the ring zone effect obtained by the mixed haemadsorption technique on thyroid cultures with sera from patients with thyroid autoimmunity were continued in a model system that permitted a controlled variation of some relatively well defined and simple antigens (human serum albumin HSA dinitrophenyl human serum albumin, DNP HSA) Varying doses of these substances were conjugated to human monolayer cultures by the aid of bis-diazotized benzidine Reactions to the antigens were examined by the mixed haem adsorption technique using rabbit antisera against HSA and DNP Ring zone effects were obtained with anti HSA sera only after prolonged immunization producing high anti HSA titres and only with a narrow range of relatively low HSA concentrations on the cultures More antibody was eluted from cultures pre incubated with ring zone serum than with corresponding concentrations of filled zone serum. Rabbit ring zone anti HSA attached to HSA conjugated cultures was traced more effectively by anti gamma and anti Fc sera than by anti L chain sera while filled zone anti HSA was traced equally effectively by all three types of antiglobulin Anti DNP sera did not produce ring zones with any concentration of DNP HSA on the cultures or with any obtainable DNP saturation on the HSA molecules On the other hand half saturation or more of DNP on the HSA molecules turned the ring zones obtained with anti HSA into filled zones. These results were in agreement with those obtained on thyroid cultures and lend further support to the hypothesis that the ring zones are due to steric hindrance effects on the antibodies in densely crowded clusters of de terminants representing several antigen specificities

In a previous study of the ring zone effect with the mixed haemadsorption technique on thyroid cultures the following main observations were made (*Jonsson & Fagraeus* 1969)

1 The ring zone effect was dependant on the arrangement of the antigenic determinants in clusters over the cell surface

2 The more coarsely spaced these clusters were the greater was the tendency to ring zone formation

3 The more numerous the antibody specificities present in the test sera the more likely were these sera to produce the ring zone effect

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These data led to the hypothesis that the ring zone effect is due to a crowding of antibodies on complex antigen clusters. Thereby a number of determinants on the attached antibodies are hidden to the indicator cells.

The present report treats results obtained with the systems human serum albumin—rabbit anti-human serum albumin (HSA—anti-HSA), human thyroglobulin—anti-human thyroglobulin and dinitrophenyl (DNP) protein conjugates—rabbit anti-DNP sera. The experiments to be described below were done partly in parallel to the previously described thyroid experiments and designed to transpose the experimental conditions into a more precisely defined model system so that independant lines of evidence were established.

The experiments were performed with the following particular questions in mind.

1. How does variation of the antigen concentration on the culture surface affect the characteristics of the reaction?
2. Can the cluster arrangement of the antigens be directly demonstrated by the immunofluorescence technique?
3. Is it of importance whether the antigen clusters contain a multitude of different antigenic specificities or of repetetive units of the same specificity? How does the number of antibody specificities affect the result?
4. Is the empty centre of the ring zone due to a too low or a too high antibody concentration?

MATERIALS AND METHODS

Antigens

Human serum albumin (HSA). The preparation obtained from Kabi AB Stockholm (Lot n o 19988) was stated by the manufacturers to contain at least 96 per cent of pure HSA. It gave one strong and two weaker precipitation lines against rabbit anti HSA. In the immunoelectrophoresis the former line was restricted to the albumin area. The latter two lines continued from the albumin area towards the pre albumin area.

Human serum albumin fragments (HSA FI). This preparation was kindly supplied by dr. C. Lapresle, Inst. Pasteur, Paris (*Lapresle and Durieux* 1957).

Bovine serum albumin (BSA). A preparation purchased from Armour Pharmaceutical Co. (England) was used.

Dog serum albumin (DSA). The preparation was kindly supplied by Kabi AB, Stockholm (Lot n o 1576).

Dinitrophenyl conjugated human serum albumin (DNP HSA) and egg albumin (DNP EA) were prepared as described below in collaboration with *Walter Schilling* B.A.

Human thyroglobulin was prepared as described in previous reports (*Jonsson et al.* 1968).

Monolayer cultures. HeLa cell, rabbit kidney and thyroid cultures were prepared in milk dilution bottles as described previously (*Jonsson et al.* 1965, *Jonsson et al.* 1968). The rabbit kidney cultures belonged to the cell line RK 13 and were kindly supplied by dr. *Pernilla Magnusson* (*Department of Virology*, Karolinska Institutet, Stockholm).

Antisera

Rabbit anti HSA sera. Rabbits were given 10 mg of HSA intramuscularly in Freunds complete adjuvant and then 2 mg without adjuvant every 14 days. Blood was drawn 15 days after the first and then 7 days after each injection. The titres obtained with three different anti HSA sera at three different stages of immunization appear from Table 1. The titres of the serum which was chosen for use in the further experiments was as follows in the mixed haemadsorption (MHT) and passive haemagglutination (TRC) tests.

Antigen	MHT	TRC
HSA	1/6400	1/25600
BSA	1/50	1/640
DSA	1/50	1/160

Rabbit anti DNP EA serum. DNP EA was prepared as described below and 1 ml of the preparation containing 5 mg of DNP EA in Freunds complete adjuvant was injected into each of a rabbit's hindlegs. After three weeks boosters of 2 mg without adjuvant were given at about monthly intervals until 22 mg had been given. The blood sample used was drawn 1 week after the last injection. The following titres were obtained with this serum against the following antigens in the mixed haemadsorption (MHT) and tanned red cell passive haemagglutination (TRC) tests.

Antigen	MHT	TRC
DNP HSA	1/3200	1/640
HSA	1/100	1/10
Ea	1/3200	1/5120

Rabbit anti BSA serum was prepared as described above for anti HSA serum It had the following relevant titres

Antigen	MHT	TRC
HSA	n d	1/40
BSA	1/800	1/20480
DSA	1/200	n d

Human anti thyroglobulin serum This serum was chosen from a group of sera obtained from patients with chronic thyroiditis Its titre against thyroglobulin in the TRC test was 1/3000000

Sheep anti rabbit immunoglobulin sera were kindly supplied by Dr *Kelus* Birmingham England Sera having the following specificities and titres were used (For details of procedure see below)

Specificity of Antiserum	TITRE against whole rabbit immunoglobulins in the MHT with rabbit anti HeLa cell serum attached to HeLa cells		TRC
	16 units	4 units	100 µg/ml
Sheep anti rabbit cl ain	1/12800	1/6400	1/40960
Sheep anti rabbit L-chain	1/3200	1/12800	1/320
Sheep anti rabbit Fc	1/400	1/1600	1/10240
Sheep anti whole IgG	1/12800	1/100	1/51200

The MHT was performed with 16 and 4 MH units of rabbit anti HeLa cell serum pre incubated on HeLa cell cultures as described in a previous report (*Jonsson & Fagraeus* 1969) When the anti HeLa cell antibodies had attached to the cells the culture was covered by agar and the various sheep anti rabbit globulin sera were allowed to diffuse from filter paper discs placed on the agar layer

The TRC test was performed with ammonium sulphate precipitated rabbit gamma globulin coated on tanned sheep erythrocytes (*Boyden* 1951) using a coating concentration of 100 µg/ml The previously described variation in MHT titre depending on the dose of conjugated antigen should be noted *Jonsson & Fagraeus* 1969)

Rabbit anti DSA was also prepared according to the schedule given above for rabbit anti HSA It had the following titres against DSA MHT – 1 3200 and TRC 1/81920

Rabbit anti thyroglobulin sera were prepared by immunizing rabbits with thyroglobulin according to the same schedule as rabbit anti HSA sera (see above) Two sera were employed one (1) producing ring zones and the other (2) producing filled zones in the mixed haemadsorption test with thyroglobulin conjugated to HeLa cells The titres against thyroglobulin were 1/102400 for both sera

Preparation of Dinitrophenyl—Human Serum Albumin (DNP HSA) and Dinitrophenyl—Egg Albumin (DNP EA)

To 150 ml of HSA solution containing 40 mg/ml was added an equal volume of potassium carbonate (K_2CO_3) of the same concentration To each of six portions was added crystalline sodium benzene sulphonate according to the following schedule

Portion	Sodium benzene sulphonate added (g)	DNP/HSA molar ratio of DNP/HSA solution (empirical values)
1	1 00	54
2	0 75	54
3	0 50	37
4	0 25	26
5	0 125	18
6	0 0625	10

The portions were kept under agitation in the dark for 24 hours and then freed from unconjugated dye residues by filtration through a Sephadex G 200 column The DNP HSA concentration of the solutions was adjusted to 20 mg/ml and the portions were kept at –20° C until used

Extinction curves were determined at 260 280 mµ (HSA) and 360 mµ (ε DNP-lysin) pH 7 4 and molar extinctions at these wave lengths were calculated for HSA DNP lysin and DNP HSA

The degree of conjugation was expressed as the molar ratio of DNP lysyl residues on each HSA molecule The molar extinction of DNP lysyl residues was assumed to be the same as for ε DNP lysin i e 17530 at pH 7 4 The molecular weight of DNP HSA was assumed to be the same as for unconjugated HSA i e 69000 The molar DNP/HSA ratio of the different portions is shown in the schedule above

DNP EA was prepared as portion 2 of DNP HSA in the schedule shown above

^{131}I Human Serum Albumin

^{131}I tagged HSA was purchased from the Amersham Radiochemical Centre England The preparations usually had an activity of about 500 µC/ml

Experimental preparations were examined in a Nuclear Chicago scintillation counter that gave 72 counts per minute for 1 µg/ml of HSA The

relationship between scintillation counts and μg of HSA was tolerably linear

Conjugation of Protein to Monolayer Cultures with BDB

Phosphate buffered saline (PBS) pH 7 4 7 0 ml, protein antigen solution 2 5 ml and 0 5 ml of a solution of bisdiazobenzidine were added in the order mentioned to flasks having a flat culture area of 44 cm² The concentration of the bisdiazobenzidine solution employed was varied between 1 and 30 μg/cm² The concentration of the protein antigen solution was varied between 1 and 300 μg/cm² After incubating the cultures for 20 minutes the reacting mixture was decanted the cultures were washed by carefully flooding them twice with PBS pH 7 4 and once with PBS pH 7 4 containing 2 per cent of normal rabbit serum The conjugated cultures were then immediately covered by an agar layer for the mixed haemadsorption test

The Mixed Haemadsorption Test

The details of the radial diffusion disc test employed in the present study has been described in earlier reports (*Fagraeus et al* 1965, *Jonsson et al* 1968, *Jonsson & Fagraeus* 1969) Standard indicator cells for rabbit antibody were described in earlier reports (*Fagraeus & Espmark* 1961, *Fagraeus et al* 1965) Indicator cells for tracing sheep anti rabbit antibody were prepared by coating human A (+) erythrocytes with 20 agglutinating units of sheep anti A and then with 20000 units of rabbit antisheep immunoglobulin serum The coating procedure was the same as described for indicator cells for rabbit antibody The sensitivity of the indicator cells was adjusted so that they indicated a minimum of 7×10^{-3} μg of antibody-N/cm²

The Indirect Immunofluorescence Technique

The details of the technique have been described earlier (*Fagraeus & Jonsson* 1971) The findings described in this report were read in a Leitz Orthoplan microscope with incident light (*Ploem* 1971) from the mercury lamp using exciter filter TAL 485 (Schott) and barrier filter K 510 Ocular and fluorimetric readings were recorded The anti rabbit fluorescein globulin conjugate (FITC) was prepared according to an earlier description (*Bergquist & Schilling* 1970) Its fluorescein/protein (F/P) molar ratio was 3 3 The conjugate was used in the dilution 1/5 Test sera were diluted 1/5

RESULTS

1 *Relationship Between the Ring Zone Effect and the Concentration and Mode of Attachment of the Antigen*

The relationship between the conjugation dose of HSA and the resulting concentration of HSA on the culture surface as measured from the radioactivity of ¹³¹I HSA is shown in Fig 1 a–c This figure also shows the relationship between the size and shape of the haemadsorption zone and the HSA dose used for conjugating this substance to HeLa cell cultures by the aid of BDB

The zones produced by both ring and filled zone sera were found to increase very rapidly

TABLE 1 *Capacity of Ring Zone Formation at Different Stages of Immunization in Sera from R… HeLa Cell Cultures with BDB 4 μg/cm² Compared to the Antibody Titre against HSA Obtained … Diff…*

Rabbit no	Immunization schedule	Serum I 15 days after 1st injection titre TRC	MH	zone 128 MH units	GP I…
Fs 546	HSA 10 mg in Fr adj every 14 d	640	F800	19/0	0
Fs 547		320	F400	19/0	1
Fs 548		2 560	F800	19/0	0
Fs 505	Hyperimmune serum to whole human plasma*				

TRC passive haemagglutination test against HSA according to *Boyden* (1951)
MH – mixed haemadsorption test against HSA conjugated to HeLa cell cultures
MH units – anti HSA dose calculated from the titre in the mixed haemadsorption test
GP – Ouchterlony gel precipitation test in the micromodification described by *Wadsworth* (1957)

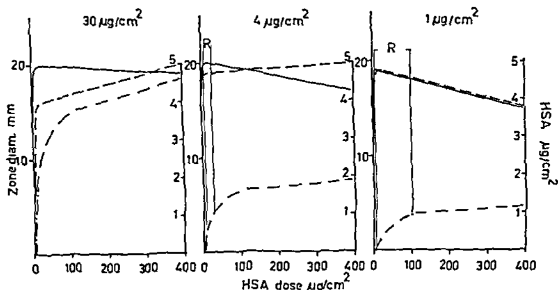


Fig 1 Relationship between size and shape of haemadsorption zone and dose of HSA used for conjugation to HeLa cell cultures Three different doses of BDB were used for conjugation (30, 4 and 1 µg/cm²) The dash-dot lines to be read by the right ordinates represent the HSA concentration attached when varying doses of HSA and BDB were used for conjugation By the left ordinate the size of the haemadsorption zone is related to the conjugation dose of HSA on the abscissa The diameters produced by a ring zone serum are indicated by solid lines and those produced by a filled zone serum by dashed lines The concentration range of HSA over which the ring zone serum gave the ring zone effect (R) is demarcated by vertical lines

in size with increasing conjugation dose of HSA up to about 10 µg/cm² where both plots relating zone size to conjugation dose of HSA bent off rather sharply The ring zone serum plot took on a nearly linear descending and the filled zone plot a slowly ascending course that was also nearly linear This applied if the two higher BDB doses

mmuni ed with HSA Shape of the Zone Produced by 128 MH Units reacting with HSA 25 µg/cm² Conjugated to as the Haemagglutination and Mixed Haemadsorption Tests and to the Number of Precipitation Lines in Gel ests

Serum 2 7 days after 2nd injection				Serum 3 7 days after 3rd injection			
titre TRC	MH	zone 128 MH units	GP lines	titre TRC	MH	zone 128 MH units	GP lines
640	F3 200	19/0	3	20 480	R12 800	19/7	5
1 280	F1 600	19/0	4	20 480	R 6 400	19/8	5
5 120	F6 400	19/0	3	20 480	R 6 400	19/9	7
				51 200	F 6 400*	19/0*	7

* This serum reacts with untreated HeLa cells but not with untreated rabbit kidney cells (RK 13) Reaction of this serum with HSA 12 5 µg/cm² conjugated to RK 13 cells with BDB 4 µg/cm² MH titre R 1600 zone produced by 128 MH units 19/13 cm

TABLE 2 *Type of Reaction against the Homologous and a Number of Heterologous Antigens Produced by 128 MH Units of some Rabbit Antiprotein Sera. The Test Dose of the Serum Was Determined against each Particular Antigen.* A Dash (–) in an Antigen Column Means that the Serum Contained less than 128 MH Units Against this Antigen. Abbreviations Are Explained in the Text*

Hyperimmune sera	Antigen						
	HSA	HSA Fract 1	DNP–HSA	DNP–EA	DSA	BSA	Human thyroglobulin
Rabbit anti HSA	19/7	19/0	19/0	–	–	–	ND
Rabbit anti HSA F1	19/0	19/0	ND	–	–	–	ND
Rabbit anti DNP EA	–	ND	19/0	19/0	–	–	ND
Rabbit anti EA	ND	ND	ND	19/0	–	–	ND
Rabbit anti DSA	ND	ND	ND	–	19/0	ND	ND
Rabbit anti thgl 1	–	–	–	–	–	–	19/8
Rabbit anti thgl 2	–	–	–	–	–	–	19/0
Human anti thgl	–	–	–	–	–	–	19/0

* Doses used for conjugation: BDB 4 µg/cm²
Antigen 12.5–25 µg/cm²

employed were used (Fig. 1 a and b). If the lowest BDB dosis was used (Fig. 1 c), the ring and filled zone plots were almost identical.

The ring zone effect was apparent over a very restricted concentration range of HSA and was more narrow with high BDB doses. It apparently corresponded to the interval 0.1–1.0 µg of attached HSA/cm.

2. *Relationship Between Ring Zone Effect and Number of Reacting Antigen Antibody Pairs*

a. Rabbit anti HSA sera had their reactivity against HSA examined at various times in the immunization course. Sera obtained early gave filled zones while sera obtained at a later stage gave ring zones. The mixed haemadsorption and tanned cell titres rose rather abruptly after three antigen injections and this was accompanied by the appearance of a ring zone effect (Table 1).

b. Anti HSA sera from hyperimmunized rabbits produced ring zones against HSA but not against HSA F1 fragments that probably carried only one determinant on each fragment. Nor could the ring zone effect be elicited with anti HSA against heterologous antigens such as bovine serum albumin (BSA) or dog serum albumin (DSA) (Table 2). Sera from rabbits immunized with BSA and DSA produced filled zones against HSA. The difference between the immunofluorescence patterns obtained when attaching HSA and DNP HSA respectively (Table 3) to the surface of suspended cells will be described in a following section.

3. *Effect of DNP Conjugation on the Antigenic Properties of HSA*

Quantitative relationships could thus be established between the titre and type of haemadsorption zone produced by anti HSA and the HSA concentration on the culture. Quite analogous relationships were found if this type of study was repeated with the monospecific antigen antibody pair HSA DNP rabbit anti DNP (Fig. 2).

The anti DNP sera were prepared by immunizing with DNP egg albumin (DNP EA). They reacted by ring zones to high titres with egg albumin. Reactions to HSA were absent or insignificant. No ring zones were ever found against DNP with these sera when the DNP/HSA molecular ratios listed on page ranging from 54 to 10 were used. Furthermore rabbit anti HSA producing ring zones with HSA alone was found to give filled zones with the same protein concentrations of DNP HSA when the aver

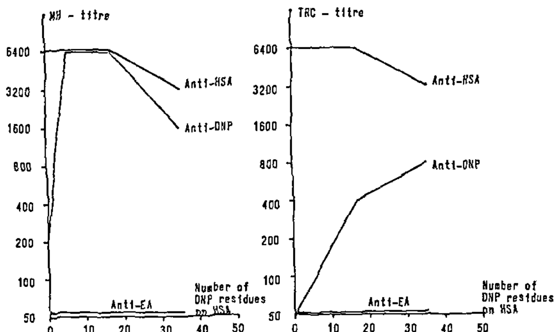


Fig 2 Reactions of rabbit antisera against human serum albumin (anti HSA) dinitrophenyl residues (anti DNP) and egg albumin (anti EA) against HSA DNP conjugates The reactions have been related to the number of DNP residues on each HSA molecule Further explanations are to be found in the text on page 170

TABLE 3 *Changes in the Immunofluorescence Pattern of HSA Coated Sheep Erythrocytes Induced by Blocking the ε Amino Groups of the HSA Lysin with DNP The Results Were Obtained with the Indirect Technique The Preparations Were Examined in a Leit. Fluorescence Microscope with Incident Illumination According to Ploem*

Sera diluted 1/5	Immunofluorescence result with: HSA only — Ocular examination: Intensity	Type of reaction	Fluorimetric count arbitrary units*	HSA conjugated with DNP DNP/HSA molar ratio 54 — Ocular examination: Intensity	Type of reaction	Fluorimetric count arbitrary units*
Rabbit anti HSA	Moderate	Diffuse staining with confluent periphery	62 5	Moderate	Granular	49 5
Rabbit anti DNP	No reaction			Weak	Granular	46 0
Normal rabbit serum	No reaction		38 5	No reaction		

* Standard error ± 4 46 counts.

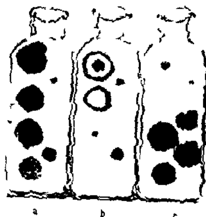
age number of DNP residues on each HSA molecule exceeded about 20 (Fig 3) *These findings were further substantiated by immunofluorescence experiments using sheep erythrocyte suspensions coated with HSA and DNP-HSA by the aid of BDB (Table 3)* The anti HSA serum used gave diffuse staining with a distinct peripheral ring against HSA If DNP HSA was used as antigen the fluorescence pattern with anti HSA serum turned into granular and its over all intensity was correspondingly reduced The same DNP HSA preparation gave a weak granular pattern if tested with anti DNP

4 *Preincubation Studies*

a As in previous experiments with human anti thyroid sera on thyroid cultures (*Jonsson & Fagraeus* 1969) a prozone corresponding to the ring zone effect could be demonstrated with the direct mixed haemadsorption technique also in the HSA-anti HSA system HeLa cell cultures carrying a HSA concentration known to support ring zone formation (BDB 3 9 $\mu g/cm$, HSA 25 $\mu g/cm^2$) were incubated with a dilution series of anti HSA serum Culture bottles were indicated immediately after incubation or after elution with phosphate buffered saline pH 2 7 The antibody contents of the eluates were determined with the radial diffusion disc test on HSA coated cultures or by estimating the radioactivity of eluates from experiments with ^{125}I tagged ring zone anti HSA serum The results appear from Table 4 The cultures incubated with prozone doses of antiserum contained more antibody than those pre incubated with dilutions in the indicable range The antibody estimates performed with the mixed haemadsorption and isotope techniques were in agreement in this respect After elution of the cultures until indicable zones had disappeared it was possible to indicate the previously non indicable centre of the ring zones with ordinary indicator cells Likewise eluates from cultures preincubated all over the surface with prozone doses of ring zone serum contained more antibody than eluates from cultures preincubated with indicable antibody doses

b *Results with Selective Anti Rabbit Immunoglobulin Sera on Pre Incubated Cultures*

HeLa cell cultures unconjugated and conjugated with HSA were pre incubated with 1 and 16 MH units of rabbit anti HeLa cell serum and rabbit ring and filled zone anti HSA serum respectively The antibodies attached to the culture at pre incubation were



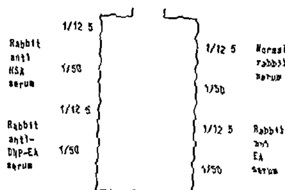


Fig 3 Rabbit anti HSA anti DNP EA and anti EA serum reacting with equivalent concentrations of a) DNP HSA b) HSA and c) EA The two dilutions of the sera employed have the same place in all three bottles and is mapped below the bottles The DNP EA serum is shown to react with DNP HSA and EA but not with HSA The figure illustrates how the ring zones produced by the anti HSA serum on HSA cultures turn into filled zones when the serum is tested against DNP HSA

TABLE 4 *Pro one Effect and Concentration of Antibody Attached in the Pro one Range The Presented Figures Are the Combined Results from Two Typical Experiments with a Ring Zone Anti HSA Serum on HeLa Cell Cultures Conjugated with HSA*

Dose of incubat on of ring zone serum MH units	Haemadsorption Before elution	Haemadsorption After elution	Number of MH units in eluate	Antibody attached as measured by isotopic technique μg/cm²
128		+ +	4	ND
64*	–	ND	ND	1 72
32	+	+ +	4	ND
16*	+	ND	ND	0 07
8	+ +	+	2	ND
4*	+ +	ND	ND	0
2	+ + +	–	0	ND
1*	+ + +	ND	ND	0

* means that the serum sample was conjugated with ^{131}I

subsequently traced by the aid of selective anti γ anti ν λ and anti Fc sera in a manner corresponding to that described previously for thyroid cultures (*Jonsson & Fagraeus* 1969) The results appear from Table 5 After pre incubation with 1 MH unit of a ring zone anti HSA serum large and distinct target zones were obtained with anti γ and anti Fc sera while the reaction with anti ν λ serum was doubtful and inconsistent After pre incubation with 4 or more MH units of a ring zone serum no reactions were obtained with anti ν λ and anti Fc sera and weak re actions with the anti γ serum After pre

TABLE 5 *Immunoglobul n Attached to HeLa Cell Cultures after Pre Incubating Them with 1 and 16 Units of a Anti HeLa Cells Serum and to HSA Conjugated HeLa Cell Cultures after Pre Incubating them with these Doses of Ring (R) and Filled (F) Zone Anti HSA Sera*

Culture	Pre incubat on Ant serum	Pre incubat on No of MH units	Anti γ serum Type	Anti γ serum Size above control	Anti L-chain serum Type	Anti L-chain serum Size above control	Anti Fc serum Type	Anti Fc serum Size above control
HeLa cell cultures unconjugated	Rabbit anti HeLa (Fs354 55)	1	R	24 0	R	25 0	R	21 0
		16	K	21 0	F	17 5	F	18 0
HeLa cell cultures conjugated with	Rabbit anti HSA R serum (Fs512)	1	K	23 0	K	Traces	K	21 0
		16	F	9 5	F	Traces	F	0 0
BDB 3 4 μg cm² HSA 25 μg cm²	Rabbit anti HSA F serum (Fs505)	1	K	24 5	K	22 0	K	19.5
		16	K	16 0	R	17 5	R	12 5

R = Ring zones
F = Filled zones
K = Target zones

incubation with a filled zone anti HSA serum or an anti HeLa cell serum large and distinct zones usually of the target or ring type were obtained with all three anti immunoglobulin sera used The results produced with filled zone sera were uninfluenced by variations of the pre incubation serum dose between 1 and 16 MH units The anti HeLa cell serum behaved like a filled zone serum in all respects

DISCUSSION

The results presented in this report are in agreement with those reported previously from experiments with thyroid cultures These experiments indicated that a certain minimal number of antibody specificities had to be present in a serum before the ring zone effect could appear This observation was further inquired into by immunization experiments. The antibody specificities in a serum should be more numerous the more complex the immunogen and thus the possibilities to produce a ring zone effect should be increased accordingly Furthermore, these possibilities should be greater the longer the immunization time employed for producing the antiserum and the more specificities contained in the test antigen The findings that ring zones could only be produced with hyperimmune sera against the homologous antigen and that anti HSA produced ring zones against HSA but not against a fragment of HSA is in strong support of this idea

More antibody was eluted from cultures pre incubated with ring zone serum dilutions than from any dilution of filled zone serum This observation is so far restricted to reactions with HSA and organ specific thyroid antigens Interspecies reactions in heterologous systems gave filled zones with higher antibody contents than ring zones (*Jonsson* &*Fagraeus* 1969) Cultures exposed to a prozone dose of antibody contained an antibody excess that was not indicable by the indicator system employed When this antibody excess was reduced by partial elution the remaining antibody was indicable Corresponding conditions could be demonstrated for the centre of the ring zones These findings are substantial evidence that the lack of indicability in the zone centre is due to a crowding of antibodies The binding affinity of the antibodies no doubt has an influence on the crowding of antibody in the zones

In agreement with the results with human sera on thyroid cultures (*Jonsson* & *Fagraeus* 1969), rabbit ring zone anti HSA attached to HSA conjugated cultures was traced more effectively by anti γ and anti Fc sera than by anti L chain sera while the corresponding filled zone anti HSA was traced about equally effectively by all three types of antiglobulin sera The ineffective tracing by all the types of antiglobulin sera after pre incubation with high doses of ring zone serum as shown in Table 4 may be explained as a negative prozone effect

The results with different concentrations of HSA on the cultures are of particular interest since in the experiments on thyroid cultures reported previously it was not possible to achieve a controlled variation of the antigen concentration The appearance of ring zones was clearly restricted to a rather narrow range of relatively low HSA concentrations Thus the ratio of antibody/antigen is of great importance for the appearance of the ring zone A narrow crowding on a restricted area with each or almost each determinant covered by an antibody seemed to promote the ring zone effect The restricted area could as in these experiments be the cell attached HSA molecule or a cluster on the thyroid cell containing presumingly many molecules with different determinants (*Jonsson* & *Fagraeus* 1969) If on the other hand the clusters were arranged close to each other or the HSA molecules were situated closely a filled zone would occur (*Jonsson* & *Fagraeus* 1969) In most cases such an antigen cluster will contain a multiplicity of specificities This may very well be the factor providing for enough crowding of antibodies to cause the ring zone since the crowding of monospecific determinants (DNP) did not give the latter effect The fact that blocking

of the ε amino groups of the HSA lysin caused the zones produced by anti HSA to change from ring to filled is in support of this idea

The number of anti DNP molecules attaching at the most at any single HSA molecule and thus the maximal number of effective DNP residues per molecule has not been determined but is certainly less than 54 However, according to preliminary immuno fluorescence experiments it appears to be more than 10 It is very probable that besides the density and number of different specificities the molecular surface relief i e the steric configuration of the antigen molecules is of importance Studies of the antigen distribution over the surface of thyroid cells grown in culture by the immunofluorescence technique (*Fagraeus* & *Jonsson* 1970) are in most respects consistent with the interpretations suggested above More detailed information on the cluster arrangement of antigenic cell surface determinants may be expected from studies with immunoferritin techniques

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BRIEF REPORTS

MYCOPLASMOSIS EXPERIMENTAL SEMINAL VESICULITIS ELECTRON MICROSCOPY OF INFECTED TISSUE

J Blom, H Erno and *A Birch-Andersen*

Mycoplasmal vesiculitis in bulls was induced by direct inoculation of *M bovigenitalium*, strain 'K', into the vesicular glands *Erno & Blom* (4) The present communication reports on the results of electron microscopical examination of infected tissue from a bull (No 185) slaughtered 40 days after inoculation of the left vesicular gland *Erno & Blom* (5) Histologically, a chronic, fibrotic, and suppurative vesiculitis was diagnosed At autopsy, secretion from the inoculated gland was found to contain 10^7 colony forming units per ml of mycoplasmas

Thirty minutes after slaughtering, the inoculated vesicular gland was removed and cut into pieces of about $3 \times 5 \times 5$ mm Initial fixation was performed at room temperature (20° C) in the formaldehyde glutaraldehyde mixture described by *Karnovsky* (9) diluted 1 1 with distilled water After a period of 2 hours the specimens were cut into $1 \times 1 \times 2$ mm strips, and fixation was continued in fresh fixative of the same osmolality for another 5 hours After a brief wash in 0 2 M sucrose in 0 1 M cacodylate buffer, pH 7 2 and storage overnight at 4° C in buffered sucrose, the tissues were postfixed for 1 hour at room temperature in 1 per cent barbiturate buffered OsO_4 pH 7 3 with 4 5 per cent sucrose added Processing for embedding in Vestopal W was carried out by the routine procedures prevalent in the laboratory Sections for electron microscopy were post stained with magnesium uranyl acetate and lead citrate For details and references see *Hovind Hougen et al* (7)

Microscopical examination of toluidine blue (0 1 per cent) stained sections (1 µm) of the vesicular gland revealed a moderate infiltration of the epithelium by neutrophilic leucocytes lymphocytes monocytes and a few plasma cells The leucocytes were also seen in the lumina of the gland together with cell debris No mycoplasmas could be detected in the sections

Electron microscopy of thin sections of the epithelium showed many of the epithelial cells in different stages of degeneration Mycoplasmas were observed extracellularly at the surface of the epithelial cells, whereas intracellular organisms were not reliably identified

The majority of mycoplasmas were observed in the lumina of the gland together with neutrophils and cellular debris A free organism is illustrated in Fig 2 In general, the pleomorphic picture characteristic of mycoplasmas was well illustrated The size and shape of mycoplasma cells varied from spherical forms measuring 0 1 µm in diameter to more elongated forms 0 2 µm in width and 0 8 µm in length The organisms were surrounded by a

Fig 1 A phagocytosed mycoplasma with a central 'vacuole" The hairy dense layer (D) is seen on the outer leaflet of the cell membrane and also on the vacuolar membrane 90 000 ×

Fig 2 A free mycoplasma clearly showing the hairy layer (D) 90 000 ×

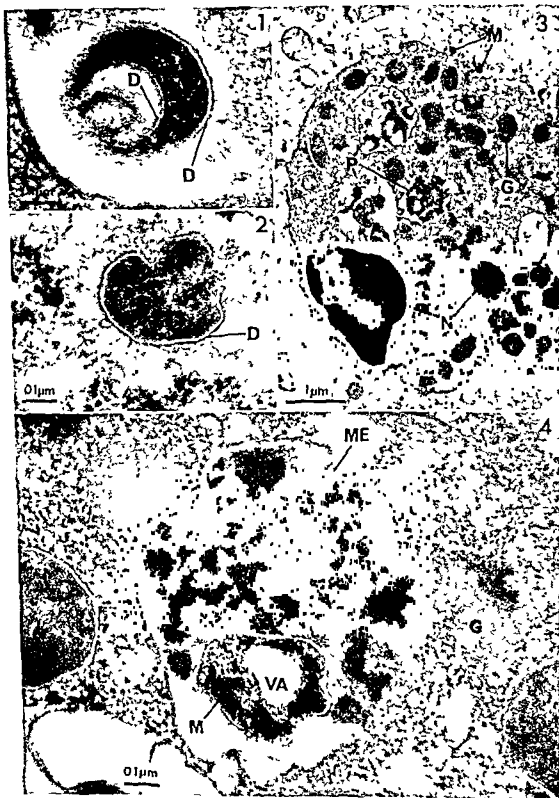
Fig 3 Part of a neutrophilic leucocyte showing a (P) 15 000 ×

Fig 4 Detail from Fig 3 The phagocytic vacuole contains a mycoplasma (M) with a central 'vacuole' (VA), some accumulations of dense material and some remnants of membranes (ME) A cytoplasmic granule (G) in the process of discharge into the vacuole is also present 90,000 ×

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1
D
D
2
D
01µm
3
M
P
G
N
1µm
4
ME
G
VA
M
01µm

[illegible] layered membrane 11–13 nm thick At some [illegible] on the [illegible] surface an electron dense layer [illegible] hair like structures was present (Figs 1 and 2 The appearance of the inte [illegible] was rather uniform and did not [illegible] of the cell The cytoplasm was so [illegible] ribosomes were difficult to [illegible] areas representative of nuclear [illegible] were [illegible] occasionally encountered in the [illegible] of micro-organisms so far studied [illegible] mycoplasmas (Figs 1 and 4), a [illegible] bounded vacuole like structure was [illegible] of the cell

[illegible] a low power electron micrograph of [illegible] a lumunar neutrophil Two lobes of [illegible] seen at the bottom of the picture [illegible] are present in the cytoplasm to- [illegible] vacuoles or phagolysosomes con [illegible] and partly ingested myco- [illegible] In [illegible] upper right hand corner, two ex [illegible] mycoplasmas are seen to be situated adjacent [illegible] plasma membrane of the cell In [illegible] of the phagolysosomes of the neutrophil [illegible] higher magnification A rather well [illegible] can be identified and many [illegible] of electron dense material are [illegible] In the organism a central empty [illegible] as described above is seen The hairy coat on the [illegible] part of the cell surface is pre served [illegible] granules of the neutrophil are seen around the vacuole (see also Fig 3) and [illegible] of the granules seems to be entering the va [illegible] probable discharge of enzymes In between the electron dense areas of the vacuole [illegible] debris are present Most likely such debris may be the remnants of previously [illegible] mycoplasmas

The general morphological features as well as [illegible] of the ultrastructural details of *M bovigenita lium* described in this study are in general agree [illegible] with the structural characteristics of myco- [illegible] studied earlier For references see *Freundt* [illegible]

Our observations are also in agreement with those made by *Jasper et al* (8) who examined mammary gland tissue of cows experimentally in fected with *M bovigenitalium* These authors failed to find mycoplasmas *in cells other than neu* trophils which is in contradiction to the observa tions by *Zucker Franklin et al* (10) who after incubation of leucocytes with mycoplasmas, demon *strated phagocytosis* by neutrophils eosinophils, monocytes and a small percentage of lymphocytes

According to the available reports, superficial extra membraneous layer on the outer leaflet of the plasma membrane has been observed in a great variety of *Mycoplasma* species (e g 1 and 3) In our preparations this layer shows a rather distinct, radiating hair like structure which also has been demonstrated earlier in cultures of a few *Myco- plasma* species (2)

The membrane bounded vacuoles observed in the present material (Figs 1 and 4) have also been demonstrated in other mycoplasmas (e g 2) In the present study, the coat of radiating hair like struc tures was also seen on the vacuolar side of the membranes bounding the vacuoles (Fig 1) ind cating that most of these represent cross-cuts of plasma membrane invaginations rather than true cytoplasmic vacuoles

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SERO-TYPING OF MYCOBACTERIA BY A NEW TECHNIQUE USING ANTIBODY GLOBULIN ADSORBED TO STAPHYLOCOCCAL PROTEIN A

Ingmar Juhlin and *Sten Winblad*

Serological typing of mycobacteria in routine work is usually performed by a tube agglutination technique proposed by *Schaefer* (1).

This method has three important disadvantages: the consumption of specific absorbed antisera is great, the mycobacteria to be typed are alive and finally, some strains cannot be typed because of spontaneous agglutination.

It has been shown earlier that gammaglobulin of IgG class will bind to the protein A on the cell walls of staphylococci by the Fc structure leaving the Fab structure oriented outwards and free to combine with antigen (2, 3, 4, 5).

In these preliminary studies specific mycobacterial typing sera were absorbed with a dense suspension of living *Staph. aureus* without protein A, strain Wood. These staphylococci were harvested from a 9 cm Petri dish with confluent growth after 2 ml 0.9 per cent *NaCl* had been added.

After centrifugation the supernate was diluted and added to a suspension of *Staph. aureus*, strain Cowan I, rich in protein A. These staphylococci had been killed by 2.5 per cent concentration of ortophenylphenol. After washing and resuspension in 0.9 per cent *NaCl* with 0.05 per cent human albumin and standardization of the density the staphylococci coated with antimycobacterial antibodies can be stored at +4° C or at −25° C without loosing the ability to react.

Type strains of mycobacteria corresponding to the antisera used were harvested from 7 H 10 medium, then suspended in 0.9 per cent *NaCl* + albumin and treated in a waterbath at 80° C for 30 minutes. The density of the suspension was then standardized.

The suspensions of staphylococci coated with different types of antimycobacterial antibodies were tested against the type strains of mycobacteria.

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Three drops of staphylococcal suspension and 3 drops of mycobacterial suspension were mixed in the wells of a disposable plastic plate which had 8×12 wells. Three drops of the mycobacterial suspension were mixed with 3 drops of 0.9 per cent NaCl as a control of spontaneous agglutination.

All strains of mycobacteria were also tested by the tube agglutination method described by *Schaefer* (1).

So far the following strains of mycobacteria and type sera have been tested:

Strains: Zemenkova IIIb 14186 – 1424 IIIb, Tudor S. V1582 IV, 2219 Altman Altman, 5433 1693 V, P 29 Scrofulaceum Bridge Scrofulaceum, PS85 Wilsson Lunning Lunning Brooks Gause, Gause 792 Gause, M. aquae 2512 Watson.

Type sera: M. avium I 568, I 393, II 573, II 570, IIIa 582, IIIb 597, IIIb 433, IV 581, IV 584, V 583, V 266, M. scrofulaceum 51 426 Chance 466, Darden 454 588 Yandle 353 Wilsson 418 506, Lunning 422, 357, Gause 441, 460, Watson 292, 389.

The plates were incubated in plastic boxes at 37° C and the agglutinations were registered at varying times from 30 minutes to 24 hours.

The preliminary results are as follows:

1. The typing by the two methods agree.
2. Even strains of mycobacteria which tended to give spontaneous agglutination and which previously could only be typed with great uncertainty were easily typed by the new method.
3. The method by which the staphylococci and mycobacteria were killed did not affect the results.
4. The amount of serum which was needed for one tube agglutination by Schaefer's method can be used for more than 10 typings by the new technique.

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OCCURRENCE AND POSSIBLE ROLE OF *MORAXELLA* SPECIES IN PIGS

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Bacteriological examination of material from 4500 *post mortem* examined suckling pigs from 17 herds resulted in the isolation of a haemolytic *Moraxella* species in 81 cases (1 8 per cent) In 43 instances *Moraxella* was isolated in pure culture and 34 pigs apparently succumbed to a generalized *Moraxella* infection Mixed infections occurred in 11 pigs and 27 isolations were from mucous membranes A tendency to a prevalence among pigs born by primiparae was found Seasonal variation with the highest frequency in the winter months was obvious The infections occurred sporadically in 13 out of 17 herds. The *Moraxella* was predominantly found among pigs less than one week old 73 of all the isolates and 94 per cent of the generalized infections were found in this age group Besides generalized infections, *Moraxella* was isolated from four cases of catarrhal pneumonia three cases of arthritis and three abscesses The morphology and the biochemical properties of the isolated strains are described In a preliminary inoculation-experiment with gnotobiotic pigs the employed *Moraxella* strain was able to establish itself in the pharynx but no clinical symptoms or pathological lesions were produced It is concluded that the described *Moraxella* species like some other gram negative related species can act as an opportunistic pathogen in pigs and occasionally cause generalized or localized infections in piglets—mainly among predisposed and debilitated individuals

During an investigation into the mortality of piglets, the bacteriological examinations repeatedly revealed bacterial strains which according to *Cowan & Steel* (1966) were identified as *Moraxella* species

The genus *Moraxella* was introduced in taxonomy by *Lwoff* in 1939 He proposed that organisms which were classified as *Haemophilus duplex* together with other related organisms should be gathered into a new genus *Moraxella*

The separation from the *Haemophilae* was based upon morphological differences and the requirement for growth factors

In 1952 *Henriksen* stated that cell morphology, a positive oxidase reaction, sensitivity to penicillin and preference for a humid atmosphere at 37°C were useful criteria in the classification of *Moraxella*

Bergey s Manual (1957) classified *Moraxella* as a genus of *Brucellaceae*, but the significance of genetic homologies in the taxonomy of bacteria gave rise to the suggestion that this genus should be incorporated into the *Neisseriaceae* (*Bovre* 1967, *Henriksen & Bovre* 1968)

Recently attention has been paid to the occurrence of variation in colony morphology

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week of age the haemolytic *Moraxella* was found predominantly in the nasal cavity and in the intestines In 4 cases *Moraxella* has been isolated from lungs with catarrhal pneumonia twice in pure culture Additionally the bacteria concerned were isolated from three cases of purulent arthritis and three abscesses

The organs from which *Moraxella* has been isolated are listed in Table 5

TABLE 5 *Moraxella Isolates from 81 Pigs*

Organ	Pure culture	Mixed culture	Total
Spleen	37	9	46
Nose		19	19
Jejunum		8	8
Lung	2	2	4
Abscess	2	1	3
Pericard um	2	1	3
Endocardium	1		1
Brain	4	2	6
Joint	2	1	3
Liver		3	3
Lymph node	1		1
	51	46	97

Bacteriology The isolated *Moraxella* species showed after overnight incubation at 37°C a colony diameter of about 1 0–1 5 mm Surface colonies on blood agar medium were surrounded by a narrow zone of beta haemolysis The colonies were circular and low convex with a glistening smooth surface and appeared greyish and semitranslucent The maximal diameter was about 3–4 mm which was reached after 3–4 days The colonies corroded the agar surface on blood agar plates and a slight tendency to spreading was noticed when the blood agar plates were incubated at 37°C in a moist chamber

The cells were gram negative to gram variable relatively plump rods coccobacilli were commonly found Diploid arrangement and short chains occurred occasionally The cells varied in size especially in old cultures and many cells contained granules The ends of the cells were distinctly rounded Biochemically they were rather inactive The biochemical properties used for identification are summarized in Table 6 The *Moraxella* was sensitive to penicillin and to neomycin nalidixic acid ampicillin streptomycin sulphonamides, oxytetracyclin chloramphenicol and furazolidone estimated by the multodisc method The mole per cent of Guanine + Cytosine (G + C) for one strain was 41 9 and for another 41 2 These values are within the range for *Moraxella* species *(Bovre 1967)*

Inoculation experiment None of the 6 experimental pigs showed any clinical symptoms or fluctuations in feed intake during the experimental period, and no gross or microscopical lesions were found at the *post mortem* examination As regards the pigs inoculated intratracheally, *Moraxella* was re isolated from the pharynx only *Moraxella* was not isolated from the pig inoculated subcutaneously or from the control pigs No antibody activity was detected by tube agglutination test

TABLE 6 *Biochemical Properties of Moraxella Strains from Piglets*

Gram	—
Mobility	—
Growth in air	+
Catalase	+
Oxidase	+
Glucose (Acid)	—
O F test	—
Growth in OF Medium	+
Growth on nutrient agar	+
Growth on MacConkey	—
Citrate utilization	—
Gelatin hydrolys s	—
Nitrate reduction	+
Urease	—
KCN	—
H S	—
Sensitivity to penicill n	+
Phenylalanine-deaminase	—

DISCUSSION

With a view to revealing these infections the essential factors were that the material received was fresh that the attached anamneses were adequate and performance of necropsi

and bacteriological investigations was systematic

The identification of these bacterial strains as belonging to the genus *Moraxella* seems justified according to the morphology of the bacterial cells in connection with a positive oxidase test, penicillin sensitivity and their biochemical inactivity A tendency to spreading and corroding effect was also observed The G + C determination furthermore supports this classification A humid atmosphere as mentioned by *Henriksen* (1952) was obtained by using fresh blood agar plates

The criteria on which *Moraxella* might be judged as the aetiological agent in infections were the presence of bacteria in pure culture or as the predominant growth along with the normal flora in an organ (*Gilardi* 1967, *Carter et al* 1970)

The organisms demonstrate a variable degree of virulence and a low degree of invasiveness The wide spread occurrence of *Moraxella* in the examined pig population (13 of 17 herds) and the symptom free presence of the organism in the nasal cavity and in the intestine suggest that *Moraxella* could be considered as a part of the normal flora of these mucous membranes Only under certain predisposing circumstances they tend to invade and cause generalized or local infections Although no enzootic occurrence in herds has been observed and in litters only one to three pigs have been proved infected series of isolates made from individual herds during the winter months may indicate periodical changes in virulence or level of contamination in the environment

The preliminary inoculation experiment has shown that the haemolytic *Moraxella* can establish itself in the pharynx The fact that the organism did not cause any clinical symptoms or pathological alterations in these experimental pigs with low immunological resistance support the impression that *Moraxella* is an organism with low pathogenicity for pigs However further experiments with other strains and other age groups of pigs are needed before the pathogenicity can be evaluated experimentally

Based on our observations it appears justified to conclude that the described *Moraxella* species like some other gram negative related species can act as an opportunistic pathogen in pigs and occasionally cause generalized infections in piglets

From a practical point of view its significance is limited and its contribution to neonatal losses in pigs seems mainly to be among predisposed and debilitated individuals

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The cooperation of *The Danish Meat Research Institute* in providing experimental animals is highly appreciated

The authors are grateful to K *Håning* DVM Dept of Microbiology and Hygiene, The Royal Veterinary and Agricultural University for determination of the mole per cent Guanine + Cytosine

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Joint	2	1	3
Liver		3	3
Lymph node	1		1
	51	46	97

Bacteriology The isolated *Moraxella* species showed after overnight incubation at 37°C a colony diameter of about 1 0–1 5 mm Surface colonies on blood agar medium were surrounded by a narrow zone of beta haemolysis The colonies were circular and low convex with a glistening smooth surface and appeared greyish and semitranslucent The maximal diameter was about 3–4 mm which was reached after 3–4 days The colonies corroded the agar surface on blood agar plates and a slight tendency to spreading was noticed when the blood agar plates were incubated at 37°C in a moist chamber

The cells were gram negative to gram variable relatively plump rods coccobacilli were commonly found Diploid arrangement and short chains occurred occasionally The cells varied in size especially in old cultures and many cells contained granules The ends of the cells were distinctly rounded Biochemically they were rather inactive The biochemical properties used for identification are summarized in Table 6 The *Moraxella* was sensitive to penicillin and to neomycin nalidixic acid ampicillin streptomycin sulphonamides oxytetracyclin, chloramphenicol and furazolidone estimated by the multodisc method The mole per cent of Guanine + Cytosine (G + C) for one strain was 41 9 and for another 41 2 These values are within the range for *Moraxella* species *(Bøvre 1967)*

Inoculation experiment None of the 6 experimental pigs showed any clinical symptoms or fluctuations in feed intake during the experimental period, and no gross or microscopical lesions were found at the *post mortem* examination As regards the pigs inoculated intratracheally, *Moraxella* was reisolated from the pharynx only *Moraxella* was not isolated from the pig inoculated subcutaneously or from the control pigs No antibody activity was detected by tube agglutination test

TABLE 6 *Biochemical Properties of Moraxella Strains from Piglets*

Gram	—
Mobil ty	—
Growth in a r	+
Catalase	+
Oxidase	+
Glucose (Acid)	—
O F test	—
Growth in OF Medium	+
Growth on nutrient agar	+
Growth on MacConkey	—
Citrate utilization	—
Gelatin hydrolysis	—
Nitrate reduct on	+
Urease	—
KCN	—
H S	—
Sensitivity to penicill n	+
Phenylalanine deaminase	—

DISCUSSION

With a view to revealing these infections the essential factors were that the material received was fresh that the attached anamneses were adequate and performance of necropsy

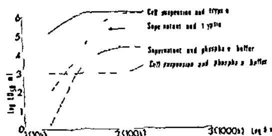


Fig 2 Effect of trypsin on toxicity suspensions and supernatants from *Clostridium botulinum* type E (toxicity in LD_{50})

linum type B and one strain of *Clostridium botulinum* type E In this experiment, the activation period was 1 hour Table 1 shows the results obtained after periods of activation of 1 hour and 168 hours, respectively The reason for choosing the prolonged activation period of 168 hours was to determine if any toxin at all could be found after such a rough treatment with proteolytic enzymes

Tryptic activation of cell suspensions of the *Clostridium botulinum* type B and type E strains increased the amount of toxin considerably compared to the cell suspensions to which buffer only was added The highest toxin level was usually reached if the cultures were examined after 4 days of incubation This is consistent with the fact that toxin-production in *Clostridium botulinum* increases during the first days of incubation and decreases thereafter As regards *Clostridium botulinum* type B, trypsinization caused only a slight increase in the supernatant as compared *with the control added buffer*, while trypsinization of the supernatant from *Clostridium botulinum* type E raised the toxin level considerably

DISCUSSION

Initially it was thought that toxin from different *Clostridium botulinum* types was monomers originating from non toxic polymer protoxins These protoxins were activated by proteases capable of breaking the polymer down to toxic monomers However, work described by *Lamanna & Sakagushi* (1971) clearly shows that botulinal toxins have another kind of structure, namely the existence of different proteins bound together in a complex In this complex, one unit is usually toxic. The different units are held together by bonds that could be broken by physical forces or by proteolytic enzymes

In this investigation trypsinization of supernatants of *Clostridium botulinum* type B did not increase the toxicity significantly, while the toxicity in the supernatants of *Clostridium botulinum* type E increased considerably As regards *Clostridium botulinum* type B, this indicates that no non toxic polymer protoxin

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Strain	Incubation period for the culture (hours)	Tryptic activation time (hours)	TOXICITY (LD_{50}/ml) Cell suspension and trypsin	Supernatant and trypsin	Cell suspension and phosphate buffer	Supernatant and phosphate buffer
Cl bot type B (strain S 64)	72	1	128000	100	1000	1
		168	18000	500	8000	10
Cl bot type B (strain 3746)	48	1	64000	10	10	0
		168	0	1	50	0
Cl bot type B (strain 8363)	48	1	64000	2000	50	1
		168	48000	0	100	1
Cl bot type E (strain 1537)	42	1	2000000	20000	1000	1000
		192	512000	100	500	500

The purpose of this investigation was to elucidate the effect of trypsin on cell suspensions and culture supernatants of non proteolytic strains of *Clostridium botulinum* types B and E, to see if activation of toxin occ the same way for these strains

MATERI

The strains u as follows

Clostridium bo
Clostridium bot
Clostridium botu
Clostridium botul

Trypticase yeast extra *et al* 1957, *Boncentre* 1 yeast extract medium f 1957) were used as growt heated for 20 min in a b by rapid cooling to expel t ated Amounts of 1000 ml w cultivation and the media 10 ml of an 18 hour-culture medium (*Sandvik* 1960) The cubated for 8 to 15 days and sa at different intervals Incubation in Mc Intosh Fildes anaerobic jars a

Crystalline trypsin was obtained (trypsin from bovine pancreas 2x cry 118 B 0880) The final concentratio used in cell suspensions and supernatant mg/ml The determination of toxicity wa out by ten fold dilutions of the reaction n and injection of these dilutions intraperito into mice The toxicity was determined as desc by *Reed* & *Munch* (1938) 0 6 M phosp buffer pH 6 2 with 0 2 per cent gelatin was us as diluent

Fifty ml were removed from the cultures for the preparation of cell suspensions These samples were centrifuged at 2000 rev/min at room temperature for 30 minutes The supernatants were decanted and kept at + 4° C until further use The cells were washed in isotonic Ringer s solution twice and recentrifuged at 2000 rev/min for 30 minutes

The cells were then resuspended in 5 ml 0 125 M isotonic phosphate buffer pH 6 0 The optical density was adjusted to the same value for all the cell suspensions

The supernatants from the different cultures were screened for proteolytic activity according to the method of *Sandvik* (1962) Trypsinization was carried out according to the following procedure

1 2 ml 0 2 mg/ml crystalline trypsin + 2 ml cell suspension
2 2 ml 0 2 m supern
3

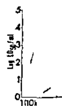


Fig 1 Effect sions and supe teolytic *Clostrid* city in LD_{50})

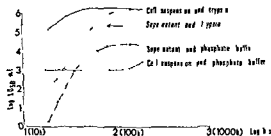


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DISCUSSION

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TABLE 1 *Influence of Tryptic Activation Period on Toxicity of Cell Suspensions and Supernatants from* Clostridium botulinum *Type B and* Clostridium botulinum *Type F* (LD_{50}/ml)

Strain	Incubation period for the culture (hours)	Tryptic activation time (hours)	TOXICITY (LD_{50}/ml) Cell suspension and trypsin	Supernatant and trypsin	Cell suspension and phosphate buffer	Supernatant and phosphate buffer
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		168	48000	500	8000	10
Cl bot type B (strain 3746)	48	1	64000	10	10	0
		168	0	1	50	0
Cl bot type B (strain 8365)	48	1	64000	2000	50	1
		168	48000	0	100	1
Cl bot type F (strain 1537)	4	1	2000000	20000	1000	1000
		192	512000	100	500	500

could be found in the supernatant, while *Clostridium botulinum* type E seems to have toxin precursors in the supernatant on which trypsin gives a marked increase in toxicity

Trypsinization of cell suspensions of *Clostridium botulinum* types B and E increased the toxicity considerably compared to the cell suspensions to which buffer was added

The length of the tryptic activation period influenced the toxicity of the cell suspensions and supernatants Activation for 7 days decreased the toxic level considerably in all strains Trypsin acting on the toxin for several days may tend to reduce the toxicity because of degradation of the toxin into smaller non toxic or weakly toxic components

The results obtained in the present experiments may, to some extent explain the difficulties involved in demonstration of toxin in food materials contaminated with *Clostridium botulinum* Proteolytic enzymes in the digestive tract may enhance the toxicity of weak toxins resulting in an intoxication The reason why toxin is not found in food materials may in these cases be that toxin production in the food materials is low and not detectable by the methods usually employed for the demonstration of toxin produced by *Clostridium botulinum*

Tryptic activation is usually used in examinations for *Clostridium botulinum* type E toxin in different foods originating from fish Trypsinization could however be used to a greater extent in the search for toxin from other types of *Clostridium botulinum*

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Acta path microbiol scand Section B 81, 2, 191–197, 1973

STUDIES ON *STACHYBOTRYS ALTERNANS* I. ISOLATION OF TOXICOGENIC STRAINS FROM FINNISH GRAINS AND FEEDS

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and Department of Plant Pathology, Agricultural Research Centre, Tikkurila, Finland

Nineteen *Stachybotrys alternans* strains isolated from animal feeds fodder or bedding and from grain for human consumption were tested for their toxin producing capacity on a mixture of wheat, oats and barley Ultraviolet light was applied during isolation of the fungi Nine out of the 19 strains were toxic to white mice in a feeding experiment of two week s duration Five selected strains were also tested by the rabbit skin test and by the feeding test with guinea pigs Clinical symptoms and patho anatomical pictures are described The reason why *S alternans* has been rarely found in grain is discussed

Toxin producing *Stachybotrys alternans* Bon [syn *S atra* Corda, *S chartarum* (Ehrenb ex Link) Hughes] fungi have been reported to cause both major outbreaks (*Sarkisov* 1954 *Yuskiv* 1968) and sporadic cases of disease in domestic animals in the Soviet-Union (*Kosevoj* 1962 *Fortushny* 1962), Hungary (*Dankó and Tanyi* 1968) and other East-European countries (*Gdovin et al* 1966 *Vachev et al* 1970) There are no corresponding reports from Western Europe However a few reports from outside Eastern Europe (*Forgacs et al* 1958 *Schumaier et al* 1963) establish the occurrence of toxicogenic *Stachybotrys* strains in nature suggesting that they possibly play a pathogenic role

S alternans which is considered saprophytic and found in soil (*Smith* 1969) grows well on materials rich in cellulose such as hay straw plant debris, clothing and paper (*Forgacs* 1962) The principal sources for stachybotryotoxicosis have been recognized to consist of contaminated straw or hay fed to the animals in rare instances heavily infected bedding (*Szabó and Széky* 1970) has been reported to be responsible for disease in pigs Stachybotryotoxicosis has been caused in horses by germinated oats, and in pigs by beans and chaff contaminated by the fungus according to *Koroleva* (1967) and *Gilazjan* (1957) respectively

Fialkov and Serebriany (1949) assigned to stachybotrys toxin the empirical formula $C\ H_{34}O_6$ or $C_{4}H_{34}O_6$ with molecular weights 430 3 or 446 3 respectively On the basis of more recent studies the formula of stachybotrys toxin is suggested to be $C_{21-25}H_{34-38}O_4$ and the toxin a steroid (*Bilaj and Pidoplichko* 1970)

It has been reported that stachybotrys toxin may affect horses cattle sheep swine (*Forgacs* 1965) and poultry (*Schumaier et al* 1963 also in field conditions as well as

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laboratory animals (*Korneev* 1948) Man is also susceptible to the toxic effects of stachybotrys toxin when handling contaminated fodder (*Gajdusek* 1953, *Sarkisov* 1954, *Spesivtseva* 1964) Stachybotrys toxin may cause local cutaneous or mucosal irritation and even necroses and changes in blood composition, especially leukopenia and thrombocytopenia, and it may have an effect on the heart resembling that of digitalis (*Palyusik 1970*) A toxic substance extracted from *S alternans* was found to be cytotoxic even at lower concentrations than aflatoxin B_1 (*Bodon and Palyusik* 1970)

Contrary to what was earlier believed, *S alternans* can rather frequently be isolated from grain and feed in Finland Testing of the isolated strains for toxigenicity was considered well motivated, especially since in several instances *S alternans* was derived from grain intended for human consumption The present paper reports on the results of such testing A preliminary work on the subject with description of the fungus has been published earlier (*Korpinen and Ylimäki* 1972)

MATERIAL AND METHODS

The 19 isolates of *S alternans* studied originated from animal feed, fodder or beddings suspected to have caused disease from grain intended for human consumption and from material gathered for phytopathological studies Table 1 states the origin of the strains The 19 isolates were found in a survey of 190 samples

Isolation of the Fungi

The fungi were isolated at room temperature on *wet cotton wool and filter paper kept in large Petri* dishes The dishes were exposed to ultraviolet light of 253 nm at room temperature for two hours daily on five days per week The fungal growth was examined by stereomicroscope If no *Stachybotrys* growth was observed within three weeks the Petri dishes were transferred into storage at 7° C temperature for later control The pure cultures were grown *on oatmeal agar*

Feeding Experiment on Mice

For the feeding experiment and for the other tests, the *S alternans* strains were cultivated for two months in 500 ml Roux flasks at room temperature on a sterilized, moist grain mixture consisting of equal parts of wheat barley and oats The grain was covered by a black layer of the fungus in 3–4 weeks This infected material was fed

TABLE 1 *Data on the Sources of Stachybotrys alternans Strains*

Fungus strain No	Isolated from	Anamnestic data
1	*Barley for human consumption*	Mycological routine investigation
2	Feed mix	Infertility of swine
3	Wheat for human consumption	Mycological routine investigation
4		– –
5	– –	– –
6	–	–
7	Feed oats	Retarded growth of swine
8	Clover seed	Mycological routine investigation
9	Hay	Respiratory symptoms in cattle
10	Commercial feed	Mycological routine investigation
11	– –	– ,–
12	Feed mix	High unspecified poultry mortality
13	Henhouse litter	–
14	–	– –
15	Red fescue	Mycological routine investigation
16	Cocksfoot	– –
17	Barley	Control of silage experiment
18	–	– –
19	Red clover seed	Mycological routine investigation

to mice. Each test group comprised six white mice (IRM) from Orion Helsinki, three of each sex weighing 25 g ± 2 g The mice were fed exclusively on the grain mixture The same grain mixture, sterilized but not inoculated with the fungi, was given to the control group After 14 days the control group and the other surviving mice were killed with ether

Preparing the Toxic Extracts

After ascertaining the results of the feeding experiment with mice, selected strains were subjected to the rabbit skin test and to the toxicity test on guinea pigs The grain mixtures infected with *Stachybotrys* and cultivated for two months were extracted with diethylether in a Soxhlet apparatus From this extract a supposedly toxic substance, Stachybotrys Toxin A, was then prepared according to the method of *Palyusik* (1970) A less complicated extracting procedure was to treat the ground grain mixture with diethylether during six days at room temperature (*Dankó and Tanyi* 1968) Both extracts were tested by the rabbit skin test For the guinea pig test toxic extract of the latter type was used

Rabbit Skin Test

The *Stachybotrys* strains No 4, 15, 16, 18, 19 and a sample of the sterilized grain were examined by the rabbit skin test The toxic substances from the test strains prepared according to *Palyusik* (1970) were dissolved in propylene glycol and injected in the shorn flank of the rabbit The corresponding toxic substances prepared according to *Dankó and Tanyi* (1968) were injected in the contralateral flank The inoculum was 0 005 ml, applied intradermally with a tuberculin syringe The extracts No 4, 15, 16 were tested on one rabbit, extracts No 18, 19 and that of the sterilized grain on another, and the solvents were tested on both animals

Toxicity Test on Guinea Pig

A quantity of 70 g of each of the grain mixtures from the strains No 4, 15, 16, 17, 18, 19 and of the sterilized grain was extracted with diethylether during six days at room temperature The ether was evaporated and the syrupy extract dissolved in 0 5 ml of 96 per cent ethanol After adding 2 ml water and evaporating the ethanol, a brown emulsion resulted, which was administered

TABLE 2 *Morbidity and Mortality in the Mice Groups Affected in Stachybotrys Feeding Experiment*

Day of test		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Group No															
6	s							4	3	3					
	d							2	1		2	1			
7	s							3	5	5					
	d							1			5				
8	s												3	5	3
	d												1		2
11	s								4	5	5	3	1		
	d										1	2	2	1	
12	s							2	3	3	5	4	4	4	3
	d							1				1			1
15	s						2	4	4	5	5	5	1		
	d									1			4	1	
16	s							5	6	4	3	2	1		
	d									2	2		1	1	
18	s		1	1	4	4	4	5	6	5	3	1			
	d									1	2	2	1		
19	s		9	3	5	5	5	4	2						
	d			1				1	2	2					

s = sick d = dead

to the guinea pigs through a stomach tube The guinea pigs were of commercial strain weighing 400 600 g One guinea pig was used for each extracted sample the material being estimated to suffice for one animal only

RESULTS

Feeding Experiment on Mice

Out of the 19 feeding groups in seven groups (No 6 7, 11, 15, 16, 18 and 19) the mice all died, in ten groups (No 1 2 3, 4, 5, 9 10, 13 14 and 17) no deaths occurred, and in two groups (No 8 and 12) three of the six animals died In the feeding groups with zero mortality, as well as in the control group, all animals remained clinically healthy throughout the test period Table 2 states the morbidity and mortality in the affected groups At the beginning of the experiment the mice ate the mouldy grain satisfactorily On the second day of the experiment the first symptoms of reduced appetite, apathy and slight irritation around the mouth were observed in the groups affected first Dehydration loss of weight severe necroses and crust formation on the lips kachexia and nervous symptoms followed The first mouse died on the third day of the experiment and the latest deaths occurred on the 14th day

Autopsy confirmed that the lips of the sick mice were completely covered by thick crusts and necrotic epithelium (Fig 1) Sometimes the paws were also covered with moderate crusts Haemorrhagic gastroenteritis was a common finding in the sick mice Haemorrhages of the cerebral meninges were seen in a few of the dead mice Bacteriologic and mycologic routine investigations failed to show any specific infections

Rabbit Skin Test

The rabbit who received the three types of extracts No 4 15 and 16 died within 20 hours after the injection During this short test period the extracts No 15 and 16 produced an oedematous reaction 1 cm in diameter at the site of the injection whereas No 4 gave no reaction Autopsy revealed dilated heart and myodegeneration



Fig 1 S alternans feeding experiment Bottom A mouse fed with sterilized grain (Control Group) Centre and top Mice fed with toxicogenic *S alternans* infected grains (Group 12

Of the extracts No 18 and 19 and that from sterile grain with which the other rabbit was injected those from the strains No 18 and 19 produced painful oedematous swellings at the injection sites the first signs appearing within 24 hours This rabbit was sacrificed on the eigth day after injection The ether extract of No 19 had caused a necrotic painful non purulent inflammatory swelling about 6 cm in diameter and 1 5 cm thick The ether extract of No 18 elicited a somewhat weaker reaction The reactions to ether extracts were slightly stronger than those to the toxic substances dissolved in propylene

glycol The extract from the sterile grain and the solvents which were injected gave no reactions

Toxicity Test on Guinea Pigs

The guinea pigs which received extracts No 15 16 18 and 19 became sluggish about one hour after administration The guinea pigs to whom extracts No 4 and extract from sterile grain were given remained healthy The animals supplied with extract No 16 and 18 died in 18 and 24 hours respectively Extracts No 15 and 19 made the animals appear sick and sluggish for a period of three to five days The autopsies of the recipients of No 16 and 18 extracts revealed a light brown brittle liver flabby myocardium and haemorrhages on the mucosa of stomach and duodenum

DISCUSSION

The name *Stachybotrys* is not often encountered in the West European mycological literature in contrast to that of Eastern Europe According to *Danko and Tanyi* (1968) *Stachybotrys alternans* is not easy to cultivate Rapidly growing and dominating fungi such as species of *Penicillium, Aspergillus Mucor, Rhizopus* and *Alternaria* often cover the growths of more slowly growing fungi Inadequate methods may thus be responsible for the failure to find *Stachybotrys* The most common routine methods used e g in mycological studies of feed samples are not favourable for the detection of *Stachybotrys* whose growth is likely to be prevented by other fungi The isolating method applied in this work is theoretically based on the finding that although any fungal spores are eventually killed by ultraviolet radiation those with dark-coloured walls are most resistant (*Smith* 1969) It has been established by this approach that *Sta* [illegible] *trys* appears quite frequently in Finnish gr[illegible]n and feed

According to *Kor*[illegible] [illegible]48 w[illegible]te mice fed with oats infected [illegible] *ernat* strains died after two to thre[illegible] and showed catarrhal haemorrhagic [illegible] *Kornee*

suggested that those mice who died after the sixth day of the experiment had ingested grains with atoxic *Stachybotrys* strains and thus died from other causes In the present feeding experiments those mice too who were merely sick after 14 days are considered to have been affected by stachybotryotoxicosis This is because the animals in question (in groups 8 and 12) displayed clinical symptoms and a patho-anatomical picture identical with those of the mice who died in the same or in other affected groups *Yushiv* (1969) classifies the *Stachybotrys* strains as strong moderate and weak toxin producers which cause the death of white mice in 5–6 7–8 and 10–14 days, respectively The results gained in this work are largely in agreement with those of *Yushu*

Neither *Yushu* nor *Korneev* mention any changes in the lips or the mouth of the sick mice It is a fact though that the crust formation and necroses of the lips which were established in the present feeding experiments are part of the classical picture of stachybotryotoxicosis in most of the animal species that have been studied (*Forgacs and Carll* 1962 *Palyusik et al* 1970 *Sarkisov* 1971) It is thus noted that the patho-anatomical picture presented by the mice of the present study agrees well with the general picture of stachybotryotoxicosis

Nine out of the 19 strains tested proved to be manifestly toxic in the conditions of the feeding experiment *Forgacs et al* (1958) found 26 toxin producing strains among 40 isolates and *Yushu* (1969) 5 among the 10 That the illness and deaths were results of a toxic effect is borne out by the sudden onset and rapid progression of the illness in part of the test groups by the quality of both clinical and autopsy findings and by the negative results of mycological and bacteriological investigations

The experiment only enabled a rough assessment of the quantitative differerences in toxin producing ability of the nine strains to be made but the existence of such variations is strongly suggested by the differences in [illegible]me of appearance of symptoms between the

groups Strain No 8 may be interpreted to have been the weakest toxin producer of all nine strains The rabbit skin test is widely used as a standard method for testing the toxicogenicity of certain fungi, especially those belonging to genera *Stachybotrys* (*Spesutseva 1964*) and *Fusarium* The results of the rabbit skin test and of the guinea pig test correlate well with those of the feeding test as regards presence or abscence of toxicity in the case of five of the strains tested The early death of one rabbit and its cardiac findings suggest the presence of a high concentration of toxic substance in the test strains (No 15 and 16) applied in this case The patho-anatomical picture presented by the guinea pigs agrees well with that described by *Korneev* (1948)

The present work confirmed the occurrence of toxicogenic *S alternans* strains in Finnish grain, feed and fodder If it is considered that *Stachybotrys* constitutes a disease problem only when growing on hay or straw, the question of stachybotryotoxicoses may be confined to those classical cases where animals eat *Stachybotrys*-infected hay or straw Since in the present work toxin producing *Stachybotrys* strains were also found in feeds (No 7 and 12), in commercial feed (No 11) and even in wheat intended for human consumption (No 6), the situation appears in a different light The findings support the opinion that mycotoxins constitute an even greater medical problem than has been imagined heretofore

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ON THE REALISTIC CLASSIFICATION AND EVALUATION OF SEROLOGY

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1 The *species* is the only category or *taxon* occurring in nature, it is a *reality* 2 The classification is orientated in a *horizontal* but not in a vertical way 3 The *species* is a group of related sero , bio- and phago-types In other words The *species* is a group of related cultures, defined by their serological biochemical and other properties 4 The *species* is not identical with a sero type 5 There are no subspecific or 'infrasubspecific categories below the *species* 6 The *sero , bio* and *phago types* are not 3 different categories but 3 different properties of the same organism belonging to a certain *species* on the basis of its antigenic structure 7 All the other categories or *taxa* such as *sub genus genus tribus familia* etc are artificial collections of *species* to facilitate the general view They do not exist in nature, but are creations of our mind 8 The *theory of reality* is valid not only for bacteria but also for all other living organisms it is a *general biological principle* 9 We cannot establish a family tree and a hierarchical system but only analyse the *species* occurring in nature 10 With regard to the *genus Salmonella* I follow the International *Salmonella* Subcommittee s classification and nomenclature based on the *Kauffmann White Schema* 11 I refuse to accept the proposal made by *W H Ewing* according to which the *genus Salmonella* should be sub-divided into 3 biochemically defined species *S cholerae suis S typhi* and *S enteritidis* I have never accepted or recommended this proposal 12 In a short review on *Escherichia* serology the following points are emphasized The determination of O K and H antigens according to the classical method is required for an exact diagnosis of *Escherichia species* 13 The determination of L , A and B antigens is necessary for an exact diagnosis of K antigens 14 These 3 antigenic groups L A and B are defined by their agglutinable agglutinin binding and agglutinogenic properties and retain their full validity 15 The *chemo types electro types* (= immunoelectrophoresis types) or other types are valuable supplements to the *sero types* 16 The *species* diagnosis cannot be established by the *chemical* analysis of antigens but only by the classical *serological* investigation 17 Through a *diagnostic antigenic scheme* we dot not aim at a complete antigenic analysis but only at a useful instrument by which to diagnose *species*

In the orthodox classification used in the International Bacteriological Code it is a matter of a *vertical* classification

Every individual is treated as belonging to a number of categories of consecutive rank and consecutively subordinate of these the *species* is the basic one The principal categories in ascending sequence are *species genus family order class division* in many *species* subspecies or varieties are distinguished in some cases subdivisions of a *species* such as strains groups serotypes variants phases and others may be recognized

It should be emphasized especially that the sero-types are designated as 'infrasubspecific

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categories and that "taxa of lower rank" are differentiated from "taxa of higher rank" All these "ranks" are regarded as *realities* Only in a review of *A Lwoff*, Institut Pasteur, Paris, I have found the conception that all categories are abstract ideas *Lwoff* wrote the following

> First categories do not exist in nature They are creations of our mind a category is the result of an arbitrary grouping'

In contrast to these two theories I have established the *theory of reality* Only the *species*, defined as groups of related sero-, bio- and phago-types, are *realities*, while all other categories are arbitrary groupings of *species* to facilitate the general view The truth is midway between the two It may be mentioned that my publication "*A new, realistic classification*" was submitted to the Zentralblatt fur Bakteriologie already in the beginning of november 1970 The review by *Lwoff* appeared in 1971 and hence it was unknown to me in 1970

According to the orthodox *vertical* classification all categories are realities but according to the modern *horizontal* classification only the *species* do exist in nature Although they are defined as groups of related sero- bio- and phago-types, they have many other properties such as morphological genetical pathogenic and other qualities

They are not 3 different categories but only 3 different properties of one and the same organism Accordingly a *species* is not identical with a sero-type but is defined exactly by its antigenic formula The serological property is only a part of the *species*

Inside a *species* such as *S paratyphi B* with the antigenic formula 1 4 5 12 b 1 2 different sero-types occur with or without the O 1 antigen and with or without the 5-antigen The *species S paratyphi B* like all other *species* consists of many sero- bio- and phago-types, *i e* of cultures which are characterized by their serological biochemical and other properties All these different cultures are identified as belonging to *one species* by their common antigenic formula of the *Kauffmann-White-Schema* 1, 4, 5, 12 b 1, 2 This is not an arbitrary but a *natural, genetic* classification The *species S paratyphi-B* is not an abstract idea, but a reality, the cause of paratyphoid B fever

This is also the behaviour of the sero-, bio- and phago-types of all other *species* such as *S typhi, S cholerae suis* etc which are arranged *horizontally* and collected in larger groups, for instance, *sub-genus, genus, tribus, familia* etc All these artificial groups are not sharply separated from one another It is arbitrary whether the Arizona group is regarded as an independent *genus Arizona* or as *sub-genus* III of the *genus Salmonella* Decisive is only the practical expediency, *i e* the insertion in the *genus Salmonella* as the Arizona-*species* can be diagnosed according to the *Kauffmann White-Schema* On the other hand, it is not arbitrary whether a certain sero-, bio- and phago-type is diagnosed as *S paratyphi-B* or *S typhi-murium* This differentiation is forced upon us by the realities in nature and no further discussion is possible

With regard to *Salmonella* classification and nomenclature I follow the International *Salmonella* Subcommittee and refuse to accept the proposal made by *H H Ewing*, Atlanta, *i e* to sub-divide the *genus Salmonella* into 3 biochemically defined 'species *S cholerae-suis, S typhi* and *S enteritidis* which is not identical with the legitimate *species S enteritidis* (= 1, 9, 12 g, m -), but is a large group comprising more than 1000 *species* Names such as *S enteritidis* ser paratyphi-B etc would only cause confusion again as *S paratyphi-B* is not the causative agent in enteritis, but in paratyphoid fever The same is valid for *S paratyphi-A S paratyphi C* and *S sendai*

I have never accepted or recommended Ewing's proposal and refer to my paper in press *On the classification and nomenclature of Salmonella-Species*

Further I should like to correct a mistake made by *S T Cowan* The name '*Kauffmann White-Schema* is not proposed by me but by the International *Salmonella* Subcommittee (1934) of which *H Schutze* London,

was chairman and *R St John Brooks* was secretary

I know from experience that it is not only bacteriologists but also specialists in classification who fail to understand my *species*-definition or find it highly difficult S T Cowan has published the following sentences

> *sero fermentative phage type* A term used by Kauffmann in his redefinition of species as *a group of related sero fermentative phage types* This statement a complicating elaboration of his earlier definition of species is not easy to understand and no enterobacteriologist of my aquaintance has been able to explain it to me

Perhaps it will help if the *species* definition is formulated as follows *The species is a group of related cultures defined by their serological, biochemical and other properties*

If we assume that we have isolated from 4 different patients with paratyphoid fever 4 *Salmonella* cultures with the following properties

culture 1 1 4 5 12 b 1,2
inositol negative d tartrate negative
phage type no 1
culture 2 4 5 12 b 1 2
inositol negative d tartrate negative
phage type no 2
culture 3 1 4 12 b 1 2
inositol positive d tartrate negative
phage-type no 3
culture 4 4 12 b 1 2
inositol negative d tartrate negative
phage type no 2

As the sero bio and phago types may occur in different combinations there are many such types of the *species S paratyphi B*

Summarizing this chapter we can conclude the following The *species* is the only category or *taxon* occurring in nature and the whole classification is orientated in a horizontal way The *species* is in contrast to all other categories not an abstract idea but a *reality* which can be diagnosed and defined exactly This *theory of reality* is valid not only for bacteria but also for all other living organisms it is a *general biological principle* I am aware that this is a *revolutionary* conception and that it will take a long time before it is generally accepted

Since the serology is playing a decisive rule in modern classification I should like to discuss the evaluation of serology in general and the serology of the *Escherichia group* especially

When I started my work on the serology of the *genus Escherichia* in 1941 there was no antigenic scheme of this large group of Gram negative enteric bacteria. The prevailing opinion was that it was not possible to determine *Escherichia* cultures by serology First I tested cultures from pathological material *e g* from acute urinary infections The O sera prepared by these cultures (*i e* by boiled cultures) agglutinated the homologous boiled cultures but not or only weakly the living cultures If these O sera were absorbed by the homologous boiled cultures all agglutinins were removed

Using the so-called OL sera prepared by living cultures both living and boiled cultures were agglutinated strongly By absorption of these OL sera by boiled cultures only the O agglutinins were removed while the L agglutinins remained in the serum Only living cultures were agglutinated by these L agglutinins These results which are confirmed by all later investigators demonstrate that there are 2 different antibodies in OL sera O and L antibodies and 2 different antigens in the bacteria O and L antigens As the binding property of L antigens is destroyed by heating at 100° C there is a distinct difference between L-antigens and the Vi antigen of *Salmonella typhi* It is well known that the binding property of the Vi antigen is preserved after heating at 100° C

An exact *species* diagnosis of *Escherichia* cultures requires not only the determination of O and H antigens but also of K antigens *i e* of L A and B antigens Therefore it is not sufficient to test only the A and B antigens but it is necessary also to determine the L antigens in a correct manner The L- A and B antigens are defined by their agglutinable agglutinin binding and agglutinogenic properties and must be diagnosed in this way

With regard to the serological technique the reader is referred to my book "*The Bacteriology of Enterobacteriaceae*", especially with regard to the determination of L, A and B-antigens

The *serological* results are not changed by the investigations on the *chemistry* of antigens but only completed The same is valid for the immunoelectrophoresis and the so-called *"electro types"*, established by this method Therefore the validity of *sero types* and of the diagnostic, antigenic scheme remain unchanged The *species* diagnosis can be done only by classical, *serological* methods

Unfortunately, the chemical nature of L antigens is unknown to-day It is possible that these antigens are polysaccharides, for example the L 9 antigen which gives a typical capsular swelling reaction But it is also possible that we have to do with proteins I do not believe that L antigens are *fimbriae* as not all cultures with L-antigen give a haemagglutination reaction as I showed already in 1948

Earlier I have described a serological variation, the *L-form variation* similar to the V-W-form variation of the Vi antigen The colonies with well developed L antigen the L plus forms, are dull and opaque, the colonies without L-antigen the L minus forms, are clear and translucent It is possible to recognize the colonies with L-antigens by the naked eye The L plus forms are O inagglutinable, but the L minus forms are O agglutinable

The O inagglutinable forms with L-antigen are more *toxic* for mice than the O agglutinable forms without L antigen Accordingly, the L-antigens play an important rôle in the active and passive immunization of mice as the L-antibodies have a protective effect It is evident from these experiments which are not cited in the literature that the L-antigens should be considered not only from a *serological*, but also from an *immunological* point of view

These results correspond to the effect of the Vi antigen of *S typhi* The immunological facts and the L-form variation speak against the conception that L-antigens are *fimbriae*

I hope very much that the chemical nature of L antigens will be established soon, although the serology of these antigens is finished and needs no further confirmation In the same way in which the chemistry of the Vi antigen of *S typhi* had no influence on the serology of this antigen, the same is valid for the *Escherichia* L-antigens For further details and literature the reader is referred to my publication *"Zur Klassifikation der Escherichia K-Antigene"*, containing the following conclusion 'There are *sero types, chemotypes* and *electro-types*' But before a *chemical* investigation is started, it is necessary to know the *serological* diagnosis of the cultures

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THE ANTIBACTERICIDAL EFFECT OF RABBIT ANTISERUM ON THE KILLING OF A SMOOTH STRAIN OF *SALMONELLA ENTERITIDIS* BY CATTLE NORMAL SERUM

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Rabbit serum that is hyperimmune to a smooth virulent strain of *Salmonella enteritidis* inhibits the bactericidal effect of normal cattle serum on the strain After fractionation on DEAE-cellulose such activity was demonstrated in the IgG antibody fraction If further purified on Sephadex G 25 and tested in immunoelectrophoresis with anti whole rabbit serum the antibactericidal fraction gave a single precipitation arc identical with the line developed with specific anti rabbit IgG serum

The complement dependent bactericidal effect of normal sera from certain animal species, notably from man and cattle, on rough mutants of *Salmonella typhimurium* 395 MS is specifically extinguished by homologous rabbit hyperimmune serum (2, 3) Fractionation of the rabbit serum on DEAE-cellulose and on Sephadex G 200 and subsequent analysis by immunoelectrophoresis and ultracentrifugation showed that the antibactericidal effect was present mainly, if not exclusively, in the IgG antibody fraction No such activity was found among the IgM antibodies (9)

Most smooth *S typhimurium* strains are resistant, however, to the bactericidal effect of serum (7) as for instance the strain *S typhimurium* 395 MS The present experiments were designed to establish whether interference by anti bacterial IgG on the complement-dependent bactericidal effect also occurs with the serum sensitive smooth enterobacteria *S enteritidis* was chosen as test organism

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MATERIAL AND METHODS

Bacterial strain *S enteritidis* IS 64 was received from the type culture collection of the National Bacteriological Laboratory (SBL), Stockholm Sweden The strain was kept at +4° C on agar slants

Immunization Bacteria grown in nutrient broth (Difco) were used as immunogens They were cultivated for 18 hr and after centrifugation washed twice in 0 15 M saline solution A suspension containing 5×10^9 ml (about 1 mg/ml dry weight) was prepared using a Turner spectrophotometer (650 nm) The bacteria were then killed by heating at 56° C for one hr The suspension was tested for sterility on nutrient agar plates Rabbits were immunized and serum was collected and handled according to a method described earlier (9) with the modification that the rabbits received 2 injections of 3 mg intravenously the last week Two different rabbit sera have been tested with quite similar results The data presented here are those obtained from one of them

Cattle serum was obtained and preserved as described before

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Purification of rabbit IgG was performed by fractionation on DEAE-cellulose as described before (9) Fractions containing the first peak were pooled and concentrated to convenient volume with poly

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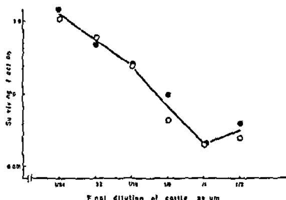


Fig 1 The bactericidal activity of normal cattle serum at different serum dilutions ○ = first day test ● = second day test The line is drawn through the geometric mean of the two tests

ethylene glycol The remainder was discarded The pooled fractions were dialysed against a triethanolamine buffer (9) overnight and passed through a column of Sephadex G 25 (Pharmacia) The flow rate was about 10 ml/hour and 3 ml fractions were again collected The absorbance at 280 nm was continuously recorded The first peak of the two obtained was concentrated with polyethylene glycol as before

Ammonium sulphate precipitation The material obtained above showed a faint precipitation line in addition to the IgG in immunoelectrophoresis To remove this the solution was treated with an equal volume of saturated ammonium sulphate solution The resultant precipitate was washed (room temperature) once with two volumes of saturated ammonium sulphate dissolved in one volume of 0 03 M *tris buffer (pH 8 4, containing 0 00015 M* Ca^{++} and 0 0005 M Mg^{++}), and finally dialysed overnight against the same buffer Only a single precipitation line then appeared with donkey anti rabbit serum (Behringwerke AG)

Bactericidal activity of cattle serum was tested according to a method described earlier (9) The bacterial concentration was 4 × 10^{3} bact /ml

The antibactericidal effect of rabbit antiserum or the IgG fraction was tested as before The concentration of *S enteritidis* IS 64 was 4 × 10^{5} bact /ml The surviving fraction and the antibactericidal titres were calculated as previously described (9) with the modification that the comparisons were made at a surviving fraction of 0 3 instead of 0 1 The curve had its steepest slope around 0 3 (Fig 2) One antibactericidal unit (AU) is defined as the protein concentration of a sample which gives a surviving fraction of 0 3

Passive haemagglutination A commercial lipo polysaccharide (LPS) from *S enteritidis* (Difco Laboratories) was treated with sodium hydroxide and used for coating sheep red blood cells (SRBC) (8) To serial two-fold dilutions of serum in portions of 0 2 ml the same volume of sensitized SRBC was added After thorough mixing the tubes were *kept at room temperature for 18 hr, when their* sedimentation patterns were observed As controls non fractionated sera and the fractions with the highest haemagglutinating titres were tested with non sensitized SRBC No titre above 2 was recorded in the controls

RESULTS

In all figures, the results of two identical experiments are recorded (termed first and second day test) The lines are drawn through the geometric mean of the two tests

The bactericidal effect of cattle serum Cattle serum had a pronounced bactericidal effect against *S enteritidis* IS 64 The effect of the serum used throughout these experiments is shown in Fig 1 where the results of two experiments are recorded

The bactericidal effect of all other cattle sera tested were similar to the one used in these experiments More than 90 per cent of the bacteria were killed in a final serum dilution of 1 8, a bactericidal effect remains also at a dilution of 1 32

The antibactericidal effect of rabbit sera In tests of the antibactericidal effect of rabbit sera, the bactericidal cattle serum was initially diluted 1 2 in tris buffer Further additions of rabbit serum and bacteria made the final con

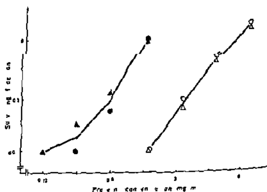


Fig 2 The antibactericidal activity of a rabbit immune serum (open symbols) and purified *IgG* (solid symbols) in relation to their protein concentrations ○ and ● = first day test △ and ▲ = *second day test* The lines are drawn through the geometric means of the tests from the two days

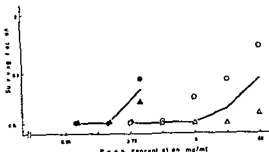


Fig 3 The antibactericidal activity of a normal rabbit serum (open symbols) and purified IgG (solid symbols) related to their protein concentrations ○ and ● = first day test △ and ▲ = second day test The lines are drawn through the geometric means of the tests from the two days

centration 1 8 The surviving fractions were determined and plotted against the protein concentration of the rabbit serum sample (Figs 2 and 3) The protein concentration of the undiluted rabbit immune serum was 73 0 mg per ml (final conc. in the test at 1 8 = 8 1 mg/ml) The antibactericidal potency of this serum was 29 AU/ml or 0 4 AU/mg (Fig 2) The effect of normal rabbit serum is shown in Fig 3 A slight antibactericidal effect appeared with rabbit normal serum The average antibactericidal potency, however, was only 1 AU/mg (or 0 02 AU/mg) since the serum was active only undiluted

The effect of rabbit IgG The sample of purified rabbit IgG prepared from the anti *S enteritidis* serum showed only one line in immunoelectrophoresis if tested with donkey anti rabbit whole serum The antibactericidal potency of the IgG was approx 2 AU/mg (Fig 2) A very slight antibactericidal effect of purified IgG from rabbit normal serum can also be seen (Fig 3)

Agglutinating activity The parent serum and the IgG were tested twice in passive haemagglutination The titre of the parent serum was 512 in one titration and 1024 in the other while that of the IgG was 128 on both occasions In relation to the protein concentration the agglutinating activity was thus enriched between 4 and 8 times Four normal rabbit sera tested had titres of 8 to 16

DISCUSSION

In a series of earlier experiments, the bactericidal effect of normal sera from man or cattle on rough mutants of *S typhimurium* 395 MS has been extinguished with rabbit antisera against the homologous mutants Antibodies with anti lipopolysaccharide specificity belonging to the IgG class are principally responsible for the phenomenon Since these antibodies activate the complement system, complement deviation has been proposed as the underlying mechanism (10, 11), but the details remain obscure

The *S typhimurium* mutants earlier investigated were of low virulence (4) As the virulent parent strain was resistant to the bactericidal action of normal serum this paper describes analogous experiments with a smooth, virulent strain of *S enteritidis* to show that the interference phenomenon also occurs with bacteria with the complete O-antigen The bactericidal effect of cattle serum on this strain was readily inhibited by rabbit anti *S enteritidis* serum and an immunoelectrophoretically pure IgG fraction derived from the latter By the purification the antibactericidal potency was increased approx five fold compared to the parent whole serum Since IgG is about one seventh of the total protein in serum, these data further support that IgG is responsible for the inhibition

The agglutinating activity of the IgG fraction was enriched 4–8 times over the parent serum This differs from results obtained with antiserum to *S typhimurium* 395 MRO, where the IgG fraction contained almost no haemagglutinating activity However, differences in lipopolysaccharide composition, extraction and coating may have influenced the results.

In addition to a direct extracellular bactericidal action, the complement system facilitates phagocytosis Since phagocytosis is stimulated by the first four complement factors

cellular killing too Thus, *Friedberg & Shilo* (5) who were looking for the bactericidal ac-

tion of the lysosomal fraction of polymorphonuclear leucocytes on different chemotypes of *Salmonella typhimurium*, found that bacteria coated with specific antisera were protected from the action of the lysosomal fraction They proposed that the protection was due to a diminished ability of "lysosomal factors" to approach their target, and was a consequence of steric or electrostatic interference by the antibodies

Thus, it is possible that the presence of antibacterial IgG may enhance the survival of intruding serum sensitive bacteria That such a mechanism exists is supported by the observation that the antibodies in chronic carriers of *S typhi* are mainly IgG (1) Furthermore, the serum from patients with chronic infections due to gram negative bacteria contains fairly frequently a factor which inhibits the bactericidal effect of normal serum (*13*) and probably is specific antibodies of the IgG class (12) However, the presence of IgG antibodies in these chronic cases may rather be a consequence of the chronic infection than its cause

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CYTOGENETICAL FINDINGS IN CHILDREN BORN TO CARRIERS OF HEPATITIS-ASSOCIATED ANTIGEN

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The chromosomes of 50 newborn infants born to healthy HAA carriers were examined No chromosomal syndrome was observed As compared with a control group the number of amodal cells was found to be significantly higher and the relative proportion of stable aberrations was also higher in the HAA group Furthermore cells with rings and dicentrics were found and the number of culture failures was significantly higher These findings suggest that infection with the HAA agent has occurred early in pregnancy even though HAA could not be detected in cord blood

Several investigations have lent support to the hypothesis of a viral aetiology of some chromosomal aberrations (7, 8, 9 15 16 33), one of these is the epidemiological study by *Collmann & Stoller* (7 33) who associated Down s syndrome with infectious hepatitis – and the chromosomal damage seen in virus infections as first shown by *Nichols* (22) Other epidemiological studies however did not show any relationship between Down s syndrome and hepatitis (6 14 17 31)

Most of the previous investigations have been concerned with infectious hepatitis (IH) By now however it is well established that long incubation hepatitis (SH) as well as IH are commonly transmitted in the population by means other than blood transfusion injection etc and some studies indicate that SH is the main type of hepatitis in adults (34)

The identification of hepatitis-associated antigen (HAA), a viruslike particle closely related to SH (27), seems to make further studies possible

Using HAA carriership as a marker of infection with the SH agent we have performed a study of HAA in pregnant women in order to determine whether or not SH infection would influence pregnancy, would be transmitted to the baby, or perhaps interact with the chromosomes of the ovum resulting in Down s syndrome or other chromosome abnormality in the children

The first part of the study has shown that the pregnancies did not imply deterioration of the healthy carrier state of the mother HAA could not be demonstrated in the cord blood and all the children remained healthy and HAA negative during a 4 to 12 months follow up (29) The results of the chromosome study in the infants are presented here

MATERIAL AND METHODS

Essentially all Danish women have a blood sample taken during pregnancy to be tested for the Wassermann reaction. During a ten month period (from

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February to October 1970, 69 978 sera from these samples, collected from all parts of the country, were investigated for the presence of HAA (28) The test was performed by gelelectrophoresis using human antisera as previously described (27) Eighty two HAA positive sera were identified A further 2 3 blood samples were obtained from these pregnant women at their regular visits and at delivery, and cord blood samples were collected

Sera from these 80 women were investigated for HAA, alanin aminotransferase, bilirubin, alkaline phosphatases and serum electrophoresis The infants were examined for clinical signs of liver disease, HAA, anti HAA and alanin aminotransferase The results of these studies together with outcome of pregnancies and case histories of the HAA positive women have been reported recently (29)

Two women were lost from the investigation

Among the 80 women, 68 delivered living children Three spontaneous abortions occurred, while 8 abortions were legally induced One child died 1 hour post partum Unfortunately no blood for chromosome studies was obtained In similarity with all the other children however, this child showed no signs of a chromosomal syndrome

The chromosomes from 50 infants were examined, 25 males and 25 females

Blood from 60 of the babies was mailed to the institute In two cases the glass was broken Cord blood was sent in 52 cases while peripheral blood was sent in 8 The culture failed in 11 out of 58 samples In the same period, 194 other blood samples were cultured by the same method in our laboratory 10 of these cultures failed

0 2 ml cord or peripheral blood was cultured for 48 to 53 hours in 7 ml McCoy's medium and 3 ml calf serum under addition of phytohaemagglutinin Colcemid (Ciba) was added two hours prior to harvesting Sodium citrate was used for hypotonic treatment and the cells were fixed in acetic methanol The slides were air dried and Giemsa stained It was planned to analyse at least 40 cells completely This however was not always possible as the quality of the preparations was below the usual standard

It was the aim of the investigators to use matched controls This turned out to be impracticable as the infants were born in different hospitals clinics or at home and a control *cord blood sample* was only obtained once, even if requests for such sample had been made

Thus, blood from infants with normal chromosomes cultured in our laboratory in the same period with the same method were used as controls This may, however give too high numbers of chromosomal aberrations in the control group as a rather large number of the infants were hospitalized and may have had infections Accordingly, the frequency of aberrations was also compared with the findings in newborns selected because they did not present any anomalies or infections they had no medication and had not been exposed to irradiations as reported recently by *Muller* (21)

A total of 2005 cells 1041 from male and 964 from female infants from the HAA group was examined 1042 cells, 616 from males and 426 from females were used as controls All cells were counted and completely analysed under the microscope Microphotographs were taken of 5 normal and of all abnormal cells and analysis was repeated on the enlargements Cells with one or more missing or additional chromosomes were scored as amodal As C_u cells (unstable aberrations) were scored cells with acentric fragments, dicentric chromosomes or ring chromosomes As C_s cells (stable aberrations) were scored cells with abnormal distribution of chromosomes in groups e g translocations rearrangements Cells which were both amodal and showed aberrations were scored separately

RESULTS

Age of Mothers

The distribution according to age of the HAA carrying mothers was compared with that of all child bearing mothers in Denmark in 1970 The HAA positive mothers were all from 17 to 33 years old This is significantly younger than ages of mothers in the normal population (p = 0 0014) (Table I)

TABLE 1 *The Distribution According to Age of Child Bearing Mothers*

Age at the time of delivery	All births in Denmark 1970	Fraction	Expected number of HAA mothers	Observed number of HAA mothers
13–33	65215	0 92109	73 69	80
34–48	5587	0 07891	6 31	0
Total	70802	1 00000	80 00	80

TABLE 2 *Cytogenetic Findings in the HAA Material*

Sex	Cord blood	Periferal blood	Total no cells examined	Chromosome count distribution 44	44	45	46	47	Amodal cells	C_u cells	C_s cells	Comments
F	+		50		2	2	45	1	5			
M	+		50		1	3	46		3	1	5	fig 1c
M	+		50		2	6	42		8			
M	culture failed	+	50	1		3	46		4	2		1 cell C_u and amodal, fig 3b, child with PKU
F	+		40			1	39		1		1	
M	+		38			5	33		4		2	
M	+		50		1	4	45		3		1	1 cell C_s and amodal
M	+		50		2	5	42		8	1		1 cell 61 chromosomes
M	+		50	1	1	9	39		11			
M	+		50	1	1	4	44		6			
M	+		49	2	1	4	31	1	7	1	1	1 cell C_s and amodal
M	+		50	3	2	6	38	1	11	1	1	2 of the cells both C_u and C_s Fig 2a
M	+		50		1	6	42	1	5	1	2	
F	+		10				10					
F	+		40	5	5	7	23		17	1		
M	+		culture failed									
M	+		40	1		3	36		4			
F	+		50		3	5	42		8			
F	+		51	1	1	4	45		6			
F		+	40		1	3	36		4			
F	+		42	1	3	2	36		5	1	1	fig 2b
F	+		28		1	1	26		2			
M	+		40	3		6	31		9			
M	+		40	4	2	7	27		13	4		
F	+		culture failed									
F	+	+	culture failed twice									
F	+		21		1	1	19		2			polyploidy, fig 3f not included in analysis
F	culture failed	+	40		2	2	36		4	2		fig 3e
F	+		40	1	4	4	31		9			
F	+		40	1	2	1	36		4			
F	+		40				40			1		fig 3a
F	+		38			1	37		1			
M		+	40		2	5	33		7			
M	glass broken											
F	+		40	1	1	7	31		9			
M		+	40	1		2	37		3			fig 3c not included in analysis

No	Sex	Cord blood	Periferal blood	Total no cells examined	Chromosome count distribution 44	44	45	46	47	Amodal cells	C_u cells	C_s cells	Comme
Au 37	F	+		40		5	3	32		8			fig 3b
Au 38	M	+		40			4	36		4			
Au 39	M		+	17	1		3	13		4			
Au 40	M	+		40	1		4	35		5			
Au 41	F	+		40			9	31		9			
Au 42	F	+		40	1	1	5	33		7	1		fig 1d
Au 43	M	+		40		5	3	32		8			
Au 44	F	+		40		2	7	31		9			f g 2c
Au 45	F	+		40		1	3	36		4		1	abnormal (not include the analysi f g 3b and
Au 46	M		+	40		1	2	37		3		1	
Au 47	M	+	contam inated	culture failed									
Au 48	M	+		culture failed									
Au 49	F	+		culture failed									
Au 50		+	+	culture failed twice									
Au 51	F			32			3	29		3	1		
Au 52	F	+		40			3	37		3	1		
Au 53	F	+	glass broken										
Au 54	M		+	40		1	1	38		3			
Au 55	M	+		19			1	18		1			
Au 56	M	+		40				40					
Au 57	F		+	40			4	36		4	1		f g 1b
Au 58	M	+		culture failed									
59	F		+	40		1		39		1	1		
^n	M	+		27			2	25		1	1	1	1 cell C_u a amodal f g

Chromosomes

The number of culture failures in the HAA group was significantly higher than in other cultures obtained during the same period ($p = 0.0001$) The modal chromosome num ber in all infants was 46 and the cytogenetic sex was in accordance with the phaenotypical sex in all cases The chromosome count distri bution and the type of aberrations observed in the HAA infants is recorded in Table 2, while findings in the controls are recorded in Table 3

The relative distribution of amodal C_u and C_s cells was heterogeneous in both materials ($p < 0.0005$) The X^2 value for the HAA material was 245 63 with 174 d f and for the controls 214 12 with 123 d f

There was no difference between the two sexes concerning the distribution of all types in either material and therefore the totals for both materials were compared (Table 4) The number of abnormal cells is significantly higher in the HAA material 14 71 per cent abnormal cells against 7 77 per cent abnormal cells in the control material ($p < 0.001$)

This difference is mainly due to a high number of amodal cells in the HAA material The difference in the number of amodal cells

TABLE 3 *Cytogenetic Findings in the Control Material*

	Sex	Cord blood	Periferal blood	Total no cells examined	Chromosome count distribution					Amodal cells	C_u cells	C_x cells	Comments
					44	44	45	46	47				
0	F		+	30		1		29		1			
0	F		+	25	1	1	4	18	1	5	2	1	
0	M		+	10			1	9		1			
0	M		+	10			1	9		1			
1	F		+	10				10					
1	M		+	10				10					
1	F		+	25			4	21		4			
1	M		+	10			1	9		1	3		
1	M		+	10				10					
1	F		+	10				10			1		
1	M		+	10				10					
1	F		+	10			2	8		2	1	1	
71	F		+	10			1	9		1			
1	F		+	10			1	9		1		1	
70	M		+	20			2	18		2	1		
71	M		+	25			2	23		2	1		
71	M		+	10			1	9		1			
71	F		+	10		1		9		1	1		cell C_u and amodal
70	M		+	30			1	29		1			
70	M		+	30				30					
70	F		+	30			2	28		2			
70	F		+	20				20					
70	F		+	30				30					
71	M		+	10				10			1		
71	M		+	10				10					
71	M		+	10				10					
1	F		+	10				10					
70	M		+	10			1	9		1			
70	M		+	20			1	19		1			
70	F		+	21		1	1	19		2		1	
70	M		+	10			1	9		1			
70	M		+	11				11					
70	M		+	50			4	46		4			
70	F		+	20			1	19		1			
70	F		+	5				5			1		
70	M		+	10				10					
cont	F	+		50			1	49		1			
70	F		+	50				50					
70	M		+	50				50					
71	M		+	50	1	1	6	42		8	1		cell C_u and amodal two dicentrics and fragment
71	F		+	50			2	48		2			
0	M		+	50			2	47	1	3	2		cell C_u and amodal
70	F		+	50			2	48		2			
70	M		+	50			5	45		5	1		
70	M		+	50			4	46		4	5		2 cells C_u and amodal

TABLE 4 *Cell Types in HAA and Control Material*

Cell type	HAA material No	HAA material %	Control material No	Control material %
All types	2005	–	1042	–
Abnormal	295	14 71	81	7 77
Amodal	254	12 67	56	5 37
C_u	19	0 95	16	1 53
C_s	17	0 85	4	0 38
C_u + Amodal	2	0 10	5	0 48
C_s + Amodal	2	0 10	0	0
61 chromosomes	1	0 05		

The percentage values are relative to the number of cells of all types

in the two materials is highly significant ($p < 0\ 001$) Regarding aberrations, no difference in the total numbers seen in the two materials was found The relative proportion of unstable and stable aberrations is given in Table 5 The relative proportion of stable aberrations in the HAA material is higher than in the control material ($p = 0\ 041$) If the materials had been homogeneous the conclusions would have been much stronger The observed significant differences between the two materials will therefore hold

Both the number and the type of aberrations in the HAA material differ significantly if compared with findings in healthy infants (21) ($p = 0\ 0001$) In the healthy newborns only 226 out of 4756 cells showed aberrations and not a single rearrangement, ring or dicentric was observed

A non random distribution of missing chromosomes was observed in our series The number of chromosomes lost from groups D and G was significantly higher than that in other groups ($p < 0\ 001$) The same pheno

TABLE 5 *Relative Proportions of Unstable and Stable Aberrations*

Cell type	HAA material No	HAA material %	Control material No	Control material %
C_u	19	53	16	80
C_s	17	47	4	20
Total	36	100	20	100

Abnormalities indicated by arrow

Fig 1 a Male infant, 45 chromosomes one F chromosome and the Y missing, an additional dicentric (Au 60)

Fig 1 b Female infant, 47 elements 3 C chromosomes missing, a dicentric and 2 acentric fragments (Au 57)

Fig 1 c Male infant, pronounced secundary constriction of B chromosome (Au 2)

Fig 1 d Female infant, 47 elements including acentric fragment (Au 42)

Fig 1 e Female infant, triradial formation of chromosome 2 and C group chromosome Chromatid break of chromosome 1 (Au 28)

menon was, however, also found in the control group A number of cells were obviously abnormal showing rings and dicentries (see Fig 3 c and e) They were not included in the analysis

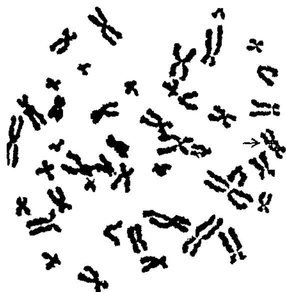
In one case, the chromosomes from an abortion induced in the 10th week of pregnancy could be examined The cells were cultured in the usual way by an ordinary tissue culture method There was an inhibition of growth as compared with other foetal cultures, but among 25 metaphases examined 19 had ad normal male karyotype one cell had a 47,XY,+G karyotype, one cell a 48,XY,+C,+G karyotype, one cell showed a rearrangement, 2 cells chromosome loss and one cell had 46,XY chromosomes with 2 additional dots, possibly rings or centric frigments The findings from the abortion tissue have not been included in the analysis

DISCUSSION

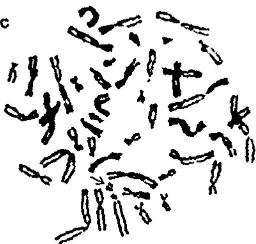
Age of Mothers

It has been found that the prevalence of healthy HAA carriers in different age groups varies considerably HAA occurs two to three times more frequently in blood donors 15–30 years of age than in those 30–50 years or older (4, 25) Similarly, the prevalence in hospitalized patients in the age group 50 years or above was only one third of that found in patients in the age group 10–30 years (30) The distribution according to age of mothers with HAA was compared with the distribution according to age of child bearing mothers

a



c



d



b



e



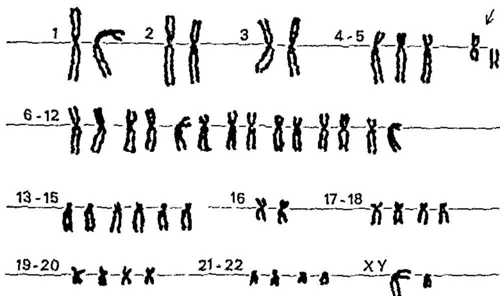


Fig 2 a Karyotype from male infant, 47 elements, B group chromosome deleted long arm and acentric fragment (Au 12)

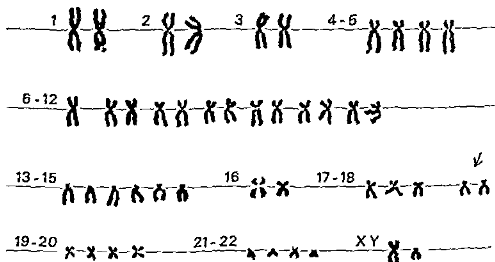


Fig 2 b Karyotype from male infant, 46 chromosomes, one 6 missing additional E group chromosome (Au 21)

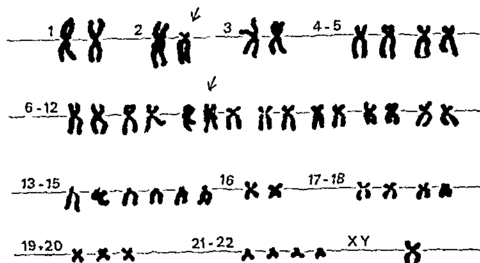


Fig 2 c Karyotype from female infant, 45 chromosomes One F chromosome missing No 2 deleted short arms (Au 45)

in Denmark and showed that the former were younger than the latter

Chromosomes

In our study no case of chromosomal syndrome was detected, especially no case of Down s syndrome Thus the findings *did not* differ significantly from those obtained in mothers in the normal population and in the age group in question Thus, the results of this study do not lend *support to the hypothesis* according to which Down s syndrome and long incubation viral hepatitis *might be* associated The number of HAA mothers is, however, small and it does not rule out the possibility that the risk could be *higher in older* HAA positive mothers

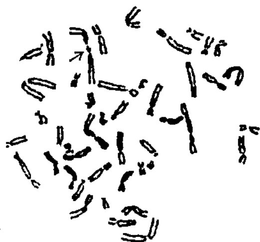
Chromosomal abnormalities after *infection* with human viruses *in vivo* and *in vitro* have been reported (2, 13 23, 32) Three types of visible virus induced chromosome abnormalities are known (21) The first may be described as single breaks they can be open breaks in the acute stage and chromosomal rearrangements in later divisions The second type is severe fragmentation or pulverization The third type involves the spindle and produces changes in chromosome number and segregation

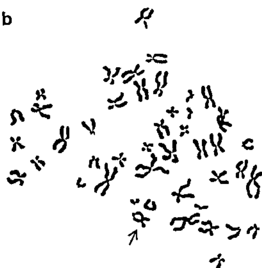
Most chromosomal studies in hepatitis have been concerned with infectious hepatitis (10, 19, 20) *In the* acute stage, chromosome breaks (20) and mitotic suppression were *observed* (19) *Later,* after one to six months, aneuploidy and rearrangements were found The chromosomes especially affected were different in the different studies *El Alfi* (10) *found* involvement of group D, *Mella* (20) of groups E, F and G, while *Matsamotu* (19) in an investigation of bone marrow found abnormalities in groups A and B

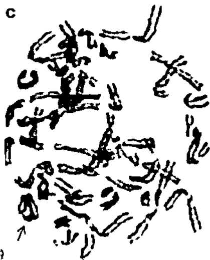
In serum hepatitis *Aya & Makino* (3) *found significantly* increased numbers of chromosome breaks in lymphocyte cultures They *observed* an excess of breaks in group D and in the paracentric segment of C group chromosomes

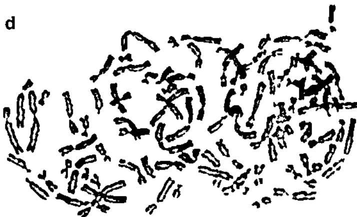
In our study, groups D and G were especially involved in chromosome loss while re-

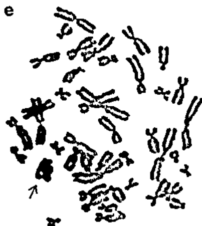
14*











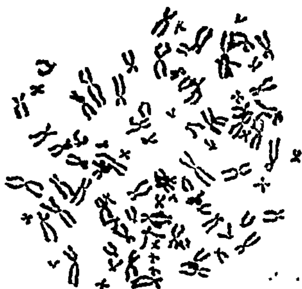


Fig 3 a Female infant 46 chromosomes pro nounced secondary constriction of chromosome No 1 (Au 31)

Fig 3 b Male infant 45 chromosomes one dicen tric (Au 4)

Fig 3 c Cell with double ring Not included in analysis (Au 45)

Fig 3 d Polyploid cell not included in analysis (Au 45)

Fig 3 e Male infant 47 elements including com plicated ringfiguration One B one C and one D and the X chromosome missing Not included in analysis (Au 36)

Fig 3 f Female infant tetraploid cell not included in analysis (Au 27)

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arrangements especially were found in groups A B and C The findings may reflect a real mechanism in chromosomal behaviour or the difficulties involved in recognizing rearrange ments of small chromosomes with a conven tional chromosome technique An analysis of such material after quinacrine staining (5, 18) or Giemsa banding (1, 26) may give fur ther information

The significantly greater loss of chromoso-mes from groups D and G may be due to an effect of the virus on the nucleolus The nu cleoli in man are organized on the short arm of acrocentric chromosomes As DNA viruses replicate in the host cell nucleolus lesions re sulting in persistence of the nucleolus have been observed

Persistence of a nucleolus shared by partner chromosomes will lead to difficulties at mito-sis causing non disjunction and chromosome loss (11)

Even though HAA was not detected in the cord blood the culture failures the high number of amodal cells and the type of aber rations observed e g rearrangements rings and dicentrics support the theory that the in-fection has occurred early in pregnancy and may have subsided at term or, alternatively may have induced a tolerant virus carrier state with an undectable serum concentration of HAA It is not known whether such in fection has significance later on in life It will be of interest to follow these HAA children also later throughout life It may elucidate whether infection with SH in early embryo genic life has any effect e g malignancy The latter has been postulated to apply to viruses in leukaemia (12)

Our thanks are due to our colleagues too numer ous to mention here for their help and to the maternity departments for their cooperation We are indebted to Mrs *Karin Hjelm Knudsen* and Mrs *Inge Frandsen* for skilful technical assistance We are indebted to Mr *Jon Stene* Lecturer, who provided statistical help and guidance

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STUDY OF AUSTRALIA—SH ANTIGEN IN DILUTED HUMAN SERUM BY ELECTRON MICROSCOPY

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The Australia SH antigen has been studied by electron microscopy of diluted sera which had not been subject to any purification procedure Australia SH antigen associated particles could be demonstrated in serum dilutions up to 1/10 000, with or without specific antiserum added Different types of aggregates depending on the relative amounts of antigen and antibody added to a sample, were observed in checkerboard titration experiments Australia SH antigen was most readily recognized in samples with about equal concentrations of antiserum and antigen-containing serum, or in samples with moderate antigen excess. For optimal results serum dilutions of 1/100 and 1/1 000 have been found most suitable Particles indistinguishable from those of the Australia SH antigen were observed in serum of an apparently healthy student selected as a negative control This serum sample showed negative reaction patterns with antiserum to the Australia SH antigen both in agar gel double diffusion tests and in complement fixation tests.

The Australia SH antigen was originally demonstrated by double diffusion experiments in agar gel (*Blumberg & Alter* 1965, *Prince* 1968) Several other methods have, however, been used for identification of this antigen such as complement fixation (*Shulman & Barker* 1969), counter immunoelectrophoresis (*Pesendorfer et al* 1970, *Prince & Burke* 1970), and electron microscopy (*Bayer et al* 1968 *Almeida et al* 1969, *Hirschman et al* 1969)

The Australia SH antigen is prevalent in serum of patients suffering from serum hepatitis (*Prince* 1968, *Giles et al* 1969 *Solaas et al* 1971), but it has also been observed in serum of apparently healthy blood donors (*Prince* 1968 *Gocke et al* 1969, *Solaas* 1970 a *Banke et al* 1971)

By electron microscopy three morphologically distinct particles have been associated with Australia-SH antigen

a Small globular particles (diameter about 20 nm) (*Prince* 1968, *Bayer et al* 1968)
b Tubular elements (diameter about 20 nm length ranging from 30–40 nm to 300 nm) (*Bayer et al* 1968)
c. Larger globular particles, often with distinct substructure (diameter about 42–46 nm) (*Dane et al* 1970, *Solaas* 1970 b)

Electron microscopy of Australia SH antigen has been performed after partial purification by centrifugation with and without addition of specific antiserum (*Bayer et al* 1968, *Almeida et al* 1969, *Hirschman et al* 1969, *Solaas* 1970 b) This paper presents the results of a methodological study on electron microscopy of whole serum with respect to the Australia SH antigen

Received 1 ix 72 Accepted 9 x 72

MATERIALS AND METHODS

Human sera Serum samples were obtained from four persons all of whom apparently healthy at the time of the bleeding

A Blood donor known to have transmitted serum hepatitis The serum contains the Australia SH antigen judged from agar gel double diffusion and complement fixation tests

B Blood donor, who by a screening was found to have Australia SH antigen in serum (*Solaas* & *Berg* 1970) He is not known to have transmitted serum hepatitis

C and D Students without Australia SH antigen in serum according to agar gel double diffusion and complement fixation experiments

Blood samples were obtained by venepuncture, allowed to clot and kept overnight at 4° C Serum was then pipetted off and stored at –40° C until examined

Antiserum to Australia SH antigen The antiserum to the Australia SH antigen was obtained from a person who five years earlier suffered from track finders hepatitis (*Berg et al* 1971) He has never been transfused No precipitating antibody to normal serum proteins could be demonstrated in this serum when it was tested against 48 normal human sera in an agar gel double diffusion experiment This antiserum rather than one from a person who has received multiple transfusions was selected to diminish the possibility of simultaneous presence of antibodies to other serum proteins

Preparation of samples for electron microscopy Prior to electron microscopy, the sera containing Australia SH antigen the control sera and the antiserum were diluted in serial tenfold dilutions with 0 85 per cent (w/v) NaCl Samples which were examined without addition of antiserum were coded and stored at –20° C until examined

Each of the dilutions of sera A B C, and D was mixed with all the dilutions of the antiserum in four checkerboard titration experiments The samples were incubated at 37° C for half an hour and tilted every tenth minute kept overnight at 4° C coded and stored at 20° C until examined in the electron microscope The coding was performed by a person who kept the code until the investigator had scored the results finally

Electron microscopy The samples were examined in the electron microscope after negative staining One drop of the sample was placed on a 200 mesh copper grid coated with 0 3 per cent (w/v) formvar and carbon and allowed to remain there for about one minute Excess fluid was then removed by touching the edge of the grid with a filter paper One drop of 3 per cent (w/v) sodium phosphotungstate solution of pH 7 4 was subsequently put on the grid for another minute and removed as described The grids were examined in a Siemens 1A electron microscope using a voltage of 80 kV and a 50 μm objective aperture The instrumental magnification was 120,000

Examination and scoring of Australia SH antigen For each sample nine squares evenly distributed throughout the grid were examined according to a pattern fixed prior to the investigation Examination of each grid lasted about 30 minutes.

Australia SH antigen was considered to be present if at least one of the following observations were made

a Aggregates of small Australia SH antigen particles only, or small particles aggregated together with tubular elements and/or larger globular particles (Fig 1, a b)

b Single tubular Australia SH structure (Fig 1, c)

c Single large globular particles with distinct substructure (Fig 1, d)

The total number of positive observations of each serum sample was recorded, as well as the number of the square on the grid where the first observation of Australia SH antigen was made

RESULTS

Checkerboard Titrations

The result of the checkerboard titration experiment with serum A, containing Australia SH antigen are presented in Table 1

In the samples located in the central areas of the checkerboard, the Australia SH antigen was found in great numbers of easily visible aggregates (Fig 1, a and b) In the samples with large antigen excess (upper right corner of the table) and/or low antibody concentration, mainly single particles

TABLE 1 *Checkerboard Titration Experiment Showing the Number of Positive Observations Obtained in Mixtures of Serum A Containing the Australia SH Antigen and Specific Antiserum*

Serum dilution*	Antiserum dilution* 1	10	10^2	10^3	10^4
1	12	5	51	37	86
10	6	59	>150§	>200§	44
10^2	2	110	200	>130§	>80§
10^3	0	4	23	120	33
10^4	0	1	8	9	5

* Reciprocal value

§ The exact numbers were difficult to count because of the presence of loose aggregates and free Australia SH antigen particles in large amounts



Fig 1 Electron micrograph showing the typical appearance of Australia SH antigen in serum *a* and *b* Aggregated Australia SH antigen particles after addition of specific antiserum *c* Tubular elements *d* Large globular particle with visible substructure (Magnification 360,000)

characteristic for the Australia-SH antigen were observed Loose aggregates predominantly consisting of tubular and large globular particles were seen in the samples with moderate antigen excess (Fig 2) An example of aggregates found in the samples with antibody excess (lower left corner of the table) is shown in Fig 3 The characteristic particles were somewhat difficult to reveal in these samples.

The result obtained by the checkerboard titration of serum B was essentially identical, but the Australia-SH antigen was more frequently observed in the serum of A than in serum of B In serum of A the Australia-SH antigen was demonstrated even in the dilution 1 10,000 By further dilution the Australia-SH antigen could not be detected In serum of B, the Australia-SH antigen was demonstrated in dilutions up to 1/1,000

Serum of C and D was selected as negative controls However, in serum obtained from C, 14 observations were made (Table 2) with single particles and aggregates (Fig 4) quite similar to those observed in the sera containing the Australia-SH antigen The Australia-SH antigen was not detected in serum obtained from D

Serial Dilutions of Sera

To evaluate whether the particles observed in serum of C were actually present in this serum or had been added to it by the antiserum, both serum of C and the antiserum



Fig 2 Electron micrograph showing aggregates of Australia-SH antigen in a sample with antigen excess (Magnification 360,000)

TABLE 2 *Checkerboard Titration Experiment Showing the Number of Positive Observations Obtained in Mixtures of Serum C, Selected as Negative Control, and Antiserum Specific to the Australia-SH Antigen (see Text)*

Serum dilution*	Antiserum dilution* 1	10	10^2	10^3	10^4
1	0	0	0	0	0
10	5	3	0	0	0
10^2	0	0	0	0	0
10^3	0	0	0	0	0
10^4	0	0	4	2	0

* Reciprocal value

as well as serum of A and B were examined in the electron microscope after tenfold serial dilutions with saline The Australia-SH antigen was found in sera of A, B, and C (Table 3), but not in the antiserum

First Observation of Australia-SH Antigen in the Samples

For practical reasons, the number of squares examined on each grid was chosen to be nine prior to the investigation It was of interest to find out whether more squares should have been examined Therefore, the

TABLE 3 *Number of Positive Observations of Australia SH Antigen after Serial Dilutions of Sera Obtained from A, B, and C, without Addition of Antiserum*

Serum dilution*	Serum sample A	B	C
10	8	4	0
10^2	6	5	0
10^3	33	0	1
10^4	4	5	1

* Reciprocal value

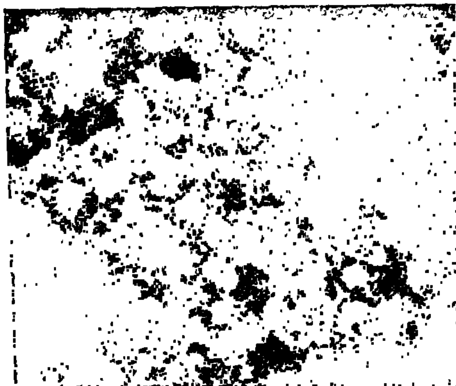


Fig 3 Electron micrograph showing aggregates of Australia SH antigen if specific antiserum was added in excess (Magnification 360,000).

number of the square where Australia-SH antigen was first observed was recorded (Table 4). In 76.4 per cent of the samples the Australia-SH antigen was found during the examination of the first three squares on a grid However, a first notation of presence of the Australia-SH antigen in a sample has been done in all the nine squares

The Australia-SH antigen was observed in the first square examined on grids, mainly in the samples with about equal concentrations of antigen-containing serum and antiserum, and in the samples with antigen excess (Table 4 a, b, and c) The table also shows that a total of 10 out of 50 samples from A and B were not found to contain Australia-SH antigen after examination of nine squares

DISCUSSION

Preparation of serum for electron microscopy with regard to Australia-SH antigen has previously been performed by gradient ultracentrifugation (*Bayer et al* 1968, *Shulman* & *Barker* 1969) or by preparative centrifugation (*Almeida et al* 1969, *Hirschman et al* 1969, *Dane et al* 1970, *Solaas* 1970 b). Using these methods, combined with addition of antiserum specific to the Australia-SH antigen, three morphologically distinct particles have been found to be associated with this antigen The results reported in the present paper show that electron microscopy of Australia-SH antigen may as well be carried out without any previous purification

The experiments indicate that the Australia-SH antigen was most frequently detected if the antigen-containing serum was diluted The study of undiluted serum in the electron microscope was hampered by aggregation of proteins on the grid In addition, undiluted serum contains large amounts of lipoproteins Especially the β-lipoproteins are very similar in size and appearance to the small Australia-

TABLE 4 *The Number of the Square on the Grid where the First Observation of Australia SH Antigen was Made in Checkerboard Titration Experiments with Mixtures of Serum of A, B and C and the Antiserum to the Australia-SH Antigen*

Serum dilution*	Antiserum dilution*				
	1	10	10	10^3	10^4
a *Serum obtained from A*					
1	3	1	1	1	1
10	1	1	1	1	1
10^2	7	1	1	1	1
10^3	–	3	2	1	1
10^4	–	7	7	1	3
b *Serum obtained from B*					
1	1	1	8	–	1
10	–	1	1	1	1
10^2	7	5	1	3	4
10^3	2		1	2	1
10^4	–	–	–	–	–
c *Serum obtained from C*					
1	–	–	–	–	–
10	1	6	–	–	–
10^2	–	–	–	–	–
10^3	–	–	–	–	–
10^4	–	–	2	9	–

* Reciprocal value
– No evidence of presence of the Australia SH antigen

SH antigen associated particles Dilutions of serum suitable for the study of Australia SH antigen by electron microscopy have been found to be 1/100 and 1/1,000 By dilution of sera with saline Australia SH antigen has been identified in dilutions up to 1/10 000 of human serum

As previously observed by *Bayer et al* (1968) and *Almeida et al* (1969), addition of serum containing antibodies to the Australia SH antigen to sera containing the Australia SH antigen causes aggregation of the particles

In the present study firm aggregates of Australia SH antigen associated particles have been observed in the samples with antiserum added in excess In these aggregates the single particles were often difficult to detect, may be because of antibody molecules covering the aggregates

The aggregates observed in the samples containing excess of Australia SH antigen were loose and predominantly consisting of tubular and large globular particles associated with the Australia SH antigen Similar aggregates have recently been found naturally occurring in sera containing Australia SH antigen by *Field & Cossart* (1971) Addition of specific antiserum to such sera made the appearance of these aggregates change, as small globular particles became the predominant form These observations made the latter authors conclude that the different morphological particles of Australia SH antigen might have different antigenic sites available and that the circulating antibody has a specificity different from that of the added antiserum

However, in the present investigation only one source of antibody to the Australia SH antigen has been used and we observed the same sort of change of the aggregates These differences may therefore also be explained by the fact that more antigenic sites are available on the larger particles than on the smaller It is possible that the former particles more readily form aggregates if the amount of antibody molecules is scarce

Serum samples obtained from two apparently healthy students were selected as negative controls, judged from negative reaction patterns against antiserum to the Australia SH antigen both in agar gel double diffusion and in complement fixation tests However, in one of these sera particles closely resembling the Australia SH antigen associated structures were observed Whether these particles really are Australia SH antigen or only similar structures which may be found in serum of healthy persons is not known

The results obtained in the checkerboard experiments show that particles associated with the Australia SH antigen were observed in the majority of the samples of A and B In samples containing undiluted serum or samples with small amounts of the antigen the Australia SH antigen associated particles were difficult to reveal, in many of these samples the presence of the antigen could

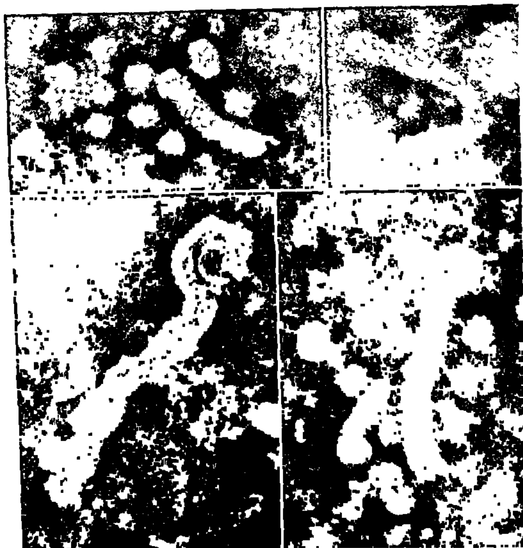


Fig 4 Electron micrograph showing Australia SH antigen like particles in serum of an apparently healthy student (C) judged to be Australia SH antigen negative on the basis of agar gel double diffusion test and complement fixation test (Magnification 360 000)

not be confirmed In such samples, examination of more than three squares on a grid often was necessary to detect the Australia-SH antigen The antigen was easily recognized in the samples where the concentration of the antigen and the antibody was about equal and in the samples with antigen excess.

Thus, the proposed method has been valuable in the study of the Australia SH antigen in serum of the blood donors However, the specificity of this test may be doubtful as particles closely resembling the Australia-SH antigen associated structures have been observed also in the serum of an apparently healthy student Using this method an extended study of the Australia-SH antigen in serum of blood donors and of patients suffering from hepatitis will be performed and published (*Solaas*, in preparation)

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Acta path microbiol scand Section B 81, 2, 227-232, 1973

CHEMOTACTIC ACTIVITY OF INTESTINAL STRANGULATION OBSTRUCTION FLUID FROM GERMFREE AND CONVENTIONAL RATS

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The chemotactic activity of rat strangulation fluid on rat polymorphonuclear leucocytes was investigated. Chemotactic activity was demonstrated in strangulation fluid from conventional as well as from germfree rats. The chemotactic substances resisted heat treatment at 56° C for 30 min. The chemotactic activity was destroyed following heat treatment at higher temperatures. Dialysis did not remove the chemotactic activity. Gel filtration chromatography on Sephadex G 100 of strangulation fluid from germfree rats resulted in fractions with three to four peaks of chemotactic activity. The possible relationship to the complement system was discussed.

Numerous investigators have been concerned about the toxic property of peritoneal exudates accumulated during intestinal strangulation obstruction. This exudate, called strangulation fluid, is sterile during the first few hours of experimental intestinal strangulation obstruction (1, 11). During the later stages of the disease, the exudate becomes heavily contaminated with bacteria of intestinal origin (1, 11). *Nemir et al* (11) noted that the picture found at death of dogs with experimental intestinal strangulation obstruction was quite different from that of bacterial peritonitis. In spite of the presence of large numbers of viable bacteria, the number of polymorphonuclear leucocytes in such exudates was hardly higher than that of the blood (4).

Previous experiments showed that sterile filtrates of rat strangulation fluid collected later than 48 hours after induction of experimental intestinal strangulation obstruction enhanced the virulence of *E. coli* towards mice (1). The filtrates did not appear to harm rat leucocytes in vitro. This was suggested by the fact that the phagocytic activity of leucocytes on *E. coli* was the same, whether the cells were preincubated in the filtrate or not (15).

The absence of frank pus in the peritoneal cavity during intestinal strangulation obstruction might suggest a lack of chemotactic stimuli in the fluid. The aim of the present study has been to elucidate the chemotactic activity of sterile strangulation fluid from conventional as well as from germfree rats on rat polymorphonuclear leucocytes, using a modification of Boyden's method (3, 6).

MATERIALS AND METHODS

Modified Gey's solution was prepared according to *Keller & Sorkin* (8). Sodium caseinate (National Biochemicals Co., Cleveland, Ohio) was dissolved

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in normal saline (3 5 per cent w/v) and sterilized by autoclaving at 120° C for 30 min

Casein Hammersten (E Merck AG, Darmstadt, Germany) was dissolved in Gey's solution (1 per cent w/v), pH 7 1

Human serum albumin (HSA), lyophilized, 96 per cent purity, was purchased from AB Kabi, Stockholm, Sweden A two per cent solution of HSA in Gey's solution was used as the cell suspension medium in all experiments

Unless stated otherwise, all media were sterilized by filtration through a Millipore filter of pore size 0 22 μ

Anti HSA Serum

Rabbits were immunized with HSA using a previously described schedule (16) The rabbit anti serum was inactivated at 56° C for 30 min, filter sterilized and stored at –20° C Immune precipitates were prepared by mixing 0 1 ml HSA (1 mg per ml in Gey's solution) with the amount of antiserum giving maximum precipitation

Animals

Germfree (GF) rats were of the CDF strain (Charles River Breeding Labs, Wilmington, Mass), reared as described by *Midtvedt & Trippestad* (10) Conventional (CONV) rats were of a local strain, kept under standard laboratory conditions

Polymorphonuclear Leucocytes (PMN)

PMN were aspirated from the peritoneal cavity of CONV rats injected with 15–20 ml of a 3 5 per cent solution of sodium caseinate 16–22 hours previously The cells were collected by centrifugation at 160 x g for 10 min at 0° C The cells were then resuspended in 20 ml of Gey's solution containing 2 per cent HSA PMN were usually pooled from three rats The number of cells was determined using a Celloscope 401 automatic cell counter (AB Lars Ljungberg & Co, Stockholm, Sweden) The volume was adjusted with Gey's solution containing 2 per cent HSA until the final cell number was 3×10^6 per ml

Serum

Serum was pooled from CONV rats and stored at –20° C

Bacteria

Escherichia coli X7 was identical with the strain used in other experiments (16) A stock suspension of washed bacteria (10^{10} per ml) was prepared in Gey's solution

Strangulation Fluid

A segment of the lower ileum was subjected to strangulation obstruction and enclosed in a plastic bag, from which strangulation fluid was collected in 24 hours portions, according to previously described techniques (1, 17) Strangulation fluid was collected from 20 CONV rats of both sexes, between 250 and 430 g of weight The fluid was pooled in three portions The „early" fluid consisted of portions collected during the first 24 hour period The „intermediate" fluid was pooled from portions collected between 24 and 48 hours after operation The „late" fluid consisted of portions collected later than 48 hours after operation.

Strangulation fluid was collected from 16 GF rats of both sexes between 80 and 120 days of age The pooled GF and CONV strangulation fluids were centrifuged, filter sterilized and stored at –20° C. Details have been described elsewhere (15 17)

Dialysis

10 ml of „intermediate" CONV strangulation fluid filtrate was dialyzed against 4 × 1000 ml of Gey's solution, pH 7 1, at 4° C As dialysis bag was used Visking tubing, of pore size approximately 24 Å The dialyzed strangulation fluid was resterilized by filtration before being tested

Heat Treatment

Aliquots of 5 ml of „intermediate" CONV strangulation fluid filtrate were incubated for 30 min in water baths of 56° C, 80° C and 100° C respectively, followed by immediate cooling to 0° C, centrifugation at 50000 x g for 30 min at 0° C and filter sterilization

Gel Filtration Chromatography

brated and eluted with Gey's solution, pH 7 1 at 4° C The flow rate was approximately 5 ml per hour Fractions of 10 ml were collected Each fraction was passed through a Millipore filter of pore size 0 22 μ, and stored at 20° C until being tested The column was calibrated by gel filtration of molecular weight marker substances, which were blue dextran, bovine serum albumin ovalbumin and bovine myoglobin

In other experiments *E coli* were used to induce chemotactic substances in normal CONV rat serum Activated serum was chromatographed on the same column Activation was done as follows 3 ml of a suspension of 10^{10} *E coli* per ml were centrifuged at 6000 x g for 10 min at 0° C The supernatant was discarded while the bacterial cells were resuspended in 3 ml of CONV rat serum The suspension was incubated at 37° C for 10 min To stop further activation, the mixture was inactivated at 56° C for 30 min The bacteria were removed by centrifugation at 6000 x g for 10 min at 0° C The supernatant was passed through a Millipore filter

of pore size 0 22 μ The filtrate was applied to the Sephadex G 100 column and chromatographed as described above

Preparation of Test Media

Strangulation fluid filtrates were tested in 10 per cent dilution to one part of the filtrate were added 9 parts of Gey s solution Fractions from gel filtra tion were tested undiluted

Positive controls (with chemotactic activity)

a) Gey s solution containing one per cent of casein Hammersten
b) 0 1 ml HSA + 0 3 ml rabbit anti HSA serum + 1 ml CONV rat serum was incubated at 37° C for 10 min then inactivated at 56° C for 30 min, finally 9 ml of Gey s solution was added

Negative control (without chemotactic activity)

One ml of CONV rat serum was incubated at 37° C for 10 min, then inactivated at 56° C for 30 min followed by the addition of 9 ml of Gey's solu tion

Chemotaxis

Chemotaxis was determined in chambers de scribed by *Boyden* (3), according to the modifica tion of *Keller* (6) The upper compartment of the chamber contained 3 6 ml of the PMN suspension (3 × 10⁵ cells per ml) The lower compartment contained 3 ml of the medium to be tested. The two compartments were separated by a Millipore filter of pore size 3 μ The chambers were incubated at 37° C for 3 hours in humid air The filters were fixed stained and mounted according to *Keller & Sorkin* (8) The cells which had migrated com pletely through the filter were counted on the lowermost filter surface at a magnification of 250 Four fields were counted on each filter, and the mean noted The results presented were the mean of at least two experiments each run in triplicate The total number of observations is indicated in the tables (n

RESULTS

From Table 1 it appears that pooled stran-gulation fluid filtrates from CONV as well as from GF rats contained substances which induced migration of 10 to 30 times as many PMN as did normal CONV rat serum

Since both early" and late' strangulation fluid contained chemotactic activity, the intermediate' portion was used for further studies (Table 2) The migration induced by strangulation fluid filtrate heat treated at 56° C for 30 min was of the same order of magnitude as the cell migration induced by

TABLE 1 *Chemotactic Activity Induced by Filter Sterilized Strangulation Fluid*

Test substance	No of PMN/field (mean±s e m)
„Early ' CONV fluid	246±34 (n = 22)
, Late CONV fluid	178±31 (n = 24)
„Late GF fluid	318±58 (n = 11)
Controls	
Rat serum	17±6 (n = 9)
HSA/anti HSA + rat serum	160±36 (n = 12)
Casein	66±11 (n = 17)

TABLE 2 *Chemotaxis Induced by Heat Treated or Dialyzed CONV Strangulation Fluid Filtrate*

	No of PMN/field (mean±s e m n=6)
56°C 30 min	242±36
80°C 30 min	16±6
100°C 30 min	30±7
Dialysis	270±29
Controls	
Untreated fluid	303±36
Rat serum	17±6
Casein	161±38

the untreated filtrate The number of cells migrating in the presence of filtrate heat treated at higher temperatures was hardly larger than of the negative control The chemotactic activity of dialyzed strangulation fluid was comparable to that of the untreated fluid filtrate

To eliminate chemotaxis induced by bac teria or bacterial products, GF strangulation fluid was used for separation on Sephadex G-100 Each fraction was tested for chemo-tactic activity in three experiments Four peaks of activity consistently appeared which corre-sponded to molecular weights of approx imately 100000 or more, 14000, 8500 and 3000 (Fig 1) The two peaks which appeared in fraction 21 and 23 were possibly part of a single broad band of activity, consisting of fractions with molecular weights between 30000 and 10000 Gel filtration of CONV rat serum activated with *E coli* resulted in a sharp peak of chemotactic activity in the molecular weight 14000 region

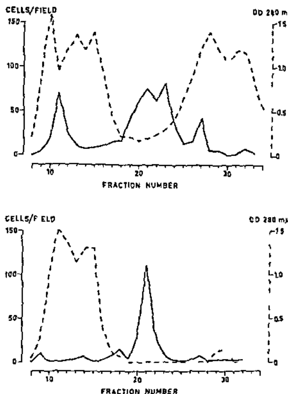


Fig 1 Upper part The elution profile of ,late GF strangulation fluid filtrate on Sephadex G 100 Lower part The elution profile of normal rat *serum* activated with *E coli*

– OD_{280} mμ —— Chemotactic activity, expressed as number of cells per field

DISCUSSION

Several investigators have shown that strangulation fluid collected during the later stages of intestinal strangulation obstruction was lethal to recipient animals when injected into the peritoneal cavity (1, 11) Positive blood cultures were found in dogs receiving a lethal dose of strangulation fluid intraperitoneally *(18) Barnett & Sampson* (2) suggested that the injection of lethal strangulation fluid caused impairment of body defence mechanisms, resulting in a lethal infection of the recipient animal

Previous studies have shown that the opsonic and bactericidal activity of rat strangulation fluid towards *E coli* was insignificant compared to the same activities of rat serum (15) Furthermore, the strangulation fluid filtrate collected more than 48 hours after the onset of strangulation obstruction, contained substances which inhibited the opsonic and bactericidal properties of serum towards the same microbe (14) The inhibitory activity could not be due to bacteria or bacterial products, since inhibitory substances were also present in GF strangulation fluid (17) The findings suggested that substances derived from the animal itself were present in strangulation fluid, and that these substances inhibited the bactericidal and opsonic components normally present in serum and tissue fluids

Two types of chemotactic substances have been described Substances which acted directly on the leucocytes in the absence of serum, were called cytotaxins (7) Other substances did not induce migration of PMN in the absence of serum but generated chemotactic substances in serum when incubated at 37° C The latter substances were called cytotaxigens (7) Many cytotaxins generated in serum by cytotaxigens have been defined as split products of complement (13, 20, 21) *Ward et al* (21) found that activation of the complement system with antigen antibody complexes generated high molecular weight cytotaxins (MW 300000) *Snyderman et al* (13) showed that endotoxin induced formation of cytotaxins in guinea pig serum, and these cytotaxins had a MW of 15000 Other cytotaxins were generated in serum following treatment with various enzymes, like trypsin (23), plasmin (19), a tissue protease (5) and lysomal enzymes from PMN (22)

It seems reasonable to assume that the cytotaxins found in strangulation fluid were of endogenous origin, since GF strangulation fluid consistently contained substantial cytotaxin activity The finding that ,late strangulation fluid filtrate inhibited the opsonic and bactericidal activity of normal serum might indicate that the filtrate contained substances which triggered the complement system The lack of opsonic and bactericidal activity in strangulation fluid might be due to consumption of complement components

The cytotaxins of strangulation fluid were non dialysable and resisted heat treatment for

30 min at 56° C In this respect they were similar to the cytotaxins generated in rabbit serum by immune complexes (3, 7) Chromatographic separation of GF strangulation fluid on Sephadex G-100 resulted in fractions with at least three peaks of activity, in contrast to the sharp peak of activity which appeared when fractions of serum activated with *E coli* were tested The present results seem to indicate that strangulation fluid contains a mixture of several cytotaxins The findings do not give conclusive evidence as to the nature of these substances It seems reasonable to assume, however, that chemotactic activity could be generated by interaction between serum factors (complement?) and substances originating in the animal itself, either from intestinal tissues or intestinal contents

As pointed out by *Mergenhagen et al* (9), many biologically active components are released following activation of the complement system by endotoxin These endogenous products may be responsible for at least part of the toxic activities of endotoxin Strangulation fluid is a heterogenous mixture of poorly defined substances which might activate the complement system in a similar way and induce formation of potent toxic substances which may contribute to the lethal effect of microbes suspended in strangulation fluid on healthy test animals

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STUDIES OF INTESTINAL AND FÆCAL SUBSTANCES FROM GERMFREE RATS INHIBITING BACTERICIDAL ACTIVITY OF RAT SERUM

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Evidence that extracts of intestinal contents and intestinal mucosa from germfree rats fed a defined diet inhibit the bactericidal activity of rat serum on *E coli* is presented and a partial chemical characterization of the active factor FIB is given FIB was present in an aqueous extract of intestinal mucosa and fæces and was non dialysable Extraction with chloroform—methanol (4 1, by vol) followed by acetone did not reduce the activity of FIB indicating that lipids were not necessary for effect The inhibiting factor resisted 100° C for 30 min FIB was also resistant to proteolysis (pronase) for 8 days, and was recovered in the resulting non-dialysable hydrolysate whereas the dialysable fraction was inactive Thiol blocking agents had no effect on FIB activity indicating that the factor was not dependent upon intact thiol groups FIB activity was enhanced by weak acid hydrolysis causing liberation of sialic acid and fucose These observations suggest that FIB is either of polysaccharide or glycoprotein nature A general inhibition of the bactericidal activity of serum exerted by polar high molecular substances was unlikely, since this effect could neither be demonstrated for the plant polysaccharides nor for the bovine glycoprotein fraction tested

The mortality rate of patients with intestinal strangulation obstruction and gangrene is about 3 times higher than the mortality rate associated with simple intestinal obstruction (13) Previous pathophysiological studies have indicated the importance of bacteria for the fatal outcome of the disease (3 4, 5) Exudate, sequestered by a strangulated loop in rats was lethal if injected into the peritoneal cavity of healthy animals, while sterile filtered fluid was non lethal (2) Recent studies have indicated that sterile filtered rat strangulation fluid has a virulence enhancing effect on *E coli*, suspended in the filtrate (8) *In vitro* experiments revealed that the filtrate inhibited the opsonic as well as the bactericidal activity of rat serum on *E coli* (15, 17) It seems reasonable to assume that the same activities might be impaired in patients with intestinal strangulation obstruction The inhibitory factor (FIB*) was found to be pre-

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* FIB = Factor causing Inhibition of Bactericidal activity of normal rat serum on *E coli*

sent also in strangulation fluid from germ free rats, indicating that FIB was of non-bacterial origin (18) *Trippestad & Midtvedt* (19) found that intestinal contents of healthy germfree rats exhibited substantial FIB activity The origin of FIB of intestinal contents remains, however, unknown In order to elucidate this, extracts of different intestinal fractions from healthy germfree rats have been tested for FIB activity In addition, investigations on the chemical nature of substances exerting FIB activity have been performed

MATERIALS AND METHODS

Reagents

All reagents were of analytical grade unless otherwise stated Toluene or thymol was used for preservation of extracts. See ref (12) for composition of Krebs Ringer buffer, containing 10 mM of glucose (KRG) Gum tragacanth was of the purity demanded by Pharmacopoea Nordica 1963 The bovine serum glycoprotein, Cohn fraction VI, was purchased from Koch Light Lab Ltd The polysaccharide from opium poppy capsula was isolated by *Wold et al* (21) The unfractionated polysaccharide material was used in this work

Animals

Germfree rats of the CDF strain (the Charles River Breeding Labs, Wilmington Mass USA) were reared as described by *Midtvedt & Trippestad* (7) Animals of both sexes more than 40 days of age (120 150 g) were used for the experiments

General Procedures

Centrifugations were performed at 30 000 x g for 30 min at 4° C Dialyser tubings were rinsed repeatedly and boiled with water for 15 min before use Solutions were concentrated under reduced pressure at temperatures below 35° C Materials were dried by lyophilization

Collection of Material

The rats were transferred to metabolic cages and fed a chemically defined diet for 5 days The diet consisted of low molecular compounds mixed with small pieces of acryl plastic (22) Fæcal material was collected from the 2nd to the 5th day using separators which disposed of the urine At the end of the 5 day period the animals were killed with ether anaesthesia After collection of contents of small intestine, caecum and colon the respective gut segments were rinsed with water The rinsing waters were combined with the respective intestinal contents The mucosal layer was separated by scraping off the inside of the intestines with glass slides After removal of the scrapings the muscular/serosal layers were obtained The material was collected under sterile conditions and stored at 20° C

Preparation of Extracts

The muscular/serosal layers were cut to small pieces by a pair of scissors Contents of small intestine, caecum colon, fæces, and the respective layers of the gut segments were purified principally in two ways by lipid extraction and ethanol precipitation

The material was extracted twice (35° C for 2 hours) with 20 times its wet weight of water saturated with thymol The extracts were clarified by centrifugation The supernatant was then passed through a Millipore prefilter (type AP 25) and finally through a Millipore filter of pore size 0 45 nm (type HA) This filtrate was tested for FIB activity (Table 1) The filtrate was concentrated and toluene was added followed by dialysis against cold water (6 × 5 l) for 2 days Both the dialysable and the non dialysable fractions were tested for FIB activity (Table 1) The non dialys

tested for FIB ac

of organic solvents in the non lipid fraction were removed by evaporation and the residue was dissolved in as little water as possible at 35° C Ethanol was added to a final concentration of 80 per cent After 24 hours at 4° C the ethanol insoluble material was centrifuged off Both the supernatant and the precipitate were tested for FIB activity (Table 1) The ethanol insoluble material was used for the present experiments if not otherwise stated

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(Table 1) was prepared in

ay as described for material HMW

Each fraction was dissolved in water and lyophilized before being tested for FIB activity Gum tragacanth the polysaccharide from *Papaver somniferum* L and the bovine glycoprotein Cohn fraction VI, were directly dissolved in water (1 mg/ml) and then tested for FIB activity

Treatment by Heat

Lyophilized fæcal material HMW dissolved in KRG (20 mg / 2 ml) was incubated in a thermostated oil bath for 30 min at 25 40 50 60 70 80 90 and 100° C At the end of the incubation period each sample was immediately placed at 20° C

Digestion with Pronase

The proteolytic digestions were carried out with the *Streptomyces griseus* protease, pronase (Koch Light Lab Ltd).

Lyophilized fecal material HMW (10 mg/ml) in 0.05 M Tris HCl buffer, pH 7.8, *containing* 0.01 M $CaCl_2$ was incubated with pronase, 0.5 *mg/ml*, at 37° C for 17 and 192 hours. The same amount of pronase was added every 24 hours (6), and the incubation continued with unaltered conditions. Controls were incubated for 192 hours either without pronase or without fecal extract. Small amounts of toluene were added to prevent bacterial growth. Activity of pronase in the incubation mixtures was controlled by testing the dialysable fractions with ninhydrin reagent (9).

The resulting hydrolysate was dialysed against cold water (6 × 5 l) for 2 days. The non-dialysable fraction was centrifuged. The precipitate was discarded. The supernatant was evaporated to dryness and dissolved in as little water as possible at 35° C. Ethanol was added to a final *concentration* of 80 per cent. After 24 hours at 4° C the precipitate formed was harvested by centrifugation. 3 fractions of each sample were tested for FIB activity (Table 2):

1. The dialysable material.
2. The non-dialysable fraction precipitated by 80 per cent of ethanol.
3. The non-dialysable alcohol soluble fraction.

Treatment with Thiol blocking Agents

Solutions of N-ethylmaleimide (Fluka) and p-chloromercuribenzoate (Sigma) were made fresh each day. Lyophilized fecal material HMW (5 mg/ml) was treated with N-ethylmaleimide 2 mM for 2 hours at room temperature. Lyophilized fecal material HMW (5 mg/ml) was similarly treated with p-chloromercuribenzoate 1 mM in sodium hydroxide solution, pH 7.6. The resulting solutions were dialysed against KRG. The non-dialysable fractions were tested for FIB activity.

Fractionation with Cetyltrimethylammonium bromide (CTAB)

Lyophilized fecal material HMW was dissolved in water to a concentration of 1 per cent. A 10 mM (0.37 per cent) aqueous solution of CTAB was added drop by drop under stirring until precipitation was completed. After 30 min at 4° C the CTAB-precipitate A (Fig. 1) was centrifuged off, washed twice with water and dissolved in ammonium formate solution (6 M) (20). Ethanol was added to a final concentration of 80 per cent. The resulting precipitate B after 24 hours at 4° C was sedimented by centrifugation and washed twice with ethanol. The precipitate B was dissolved in

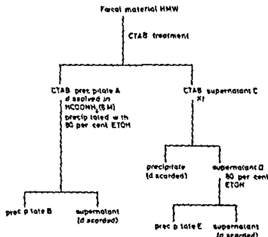


Fig. 1. Scheme for treatment with cetyltrimethylammonium bromide (CTAB) of fecal material HMW.

water and toluene was added, followed by dialysis against cold water (6 × 5 l) for 2 days.

Potassium iodide was added to the CTAB-supernatant C to eliminate excess of precipitant (1). After centrifugation ethanol was added to the supernatant D to a final concentration of 80 per cent. After 24 hours at 4° C the precipitate E was centrifuged off and washed twice with ethanol, dissolved in water and dialysed after addition of toluene against cold water (6 × 5 l) for 2 days.

The precipitated fractions B and E (Fig. 1) were lyophilized and tested for FIB activity, respectively (Table 3).

Acid Hydrolysis

Partial acid hydrolysis of fecal material HMW was effected with an aqueous suspension of Dowex 50 (H^+) at 100° C for 1 hour. The resulting non-dialysable fraction was tested for FIB activity. The material was examined by paper chromatography after acid hydrolysis. For investigation of neutral sugars the material was subjected to hydrolysis with 2 N hydrochloric acid at 100° C for 2.5 hours and for investigation of sialic acids to treatment with an aqueous suspension of Dowex 50 (H^+) at 100° C for 1 hour. For investigation of amino sugars 4 N hydrochloric acid at 100° C for 6–8 hours under nitrogen was used (22).

Paper chromatography was carried out on Whatman No. 1 filter paper in the following solvent systems by vol.: n-Butanol : pyridine : water 6 : 4 : 3. Ethyl acetate : acetic acid : formic acid : water 18 : 3 : 1 : 4. Compounds were located on chromatograms with the following reagents: Aniline oxalate (reducing sugars), ninhydrin (amino sugars) and periodate-thiobarbituric acid (sialic acids) (19).

Serum

Normal serum was obtained from conventional rats and handled as described earlier (16) Sera from several rats were pooled

Bacteria

The strain of *E coli*, and the techniques of culture and labelling with ³ P used, were described earlier (16)

Test for FIB Activity

a Preparation and standardization of test solutions Except otherwise mentioned toluene was added to each sample followed by dialysis against KRG for 24 hours After evaporation of toluene, each sample was passed through a Millipore filter (type HA), and thereafter diluted with water to contain 1 mg lyophilized material/ml before being added to the pre incubation medium

b Pre incubation of media 1 volume of test solution, 7 volumes of KRG, and 1 volume of serum were mixed in this order at 0° C Two control media were employed in each experimental series One of the controls contained 9 volumes of KRG and no serum (control without serum) The second control contained 8 volumes of KRG and 1 volume of serum (control with serum) Each medium was pre incubated at 37° C for 60 min, then immediately cooled to 0° C

c Addition of ^{32}P labelled bacteria to the pre incubated media To each of the pre incubated media was added 1 volume of a suspension of ^{32}P labelled *E coli* in KRG (10^{10}/ml) at 0° C The final test suspensions contained 10^9 bacteria 0 1 mg lyophilized material and 10 per cent of serum/ml

d Determination of released label from bacteria into the medium Aliquots of 2 ml of the final test suspension were incubated at 37° C for 15 min The suspensions were then centrifuged at 6 000 x g for 10 min at 0° C The radioactivity released to the [illegible] was determined by liquid scintillation [illegible] The release of label was expressed as cpm/[illegible] For a more detailed description of the m[illegible] e ref (16)

Presentation [illegible]ts

The respecti[illegible]nal fractions and fæces from 4 rats were tested [illegible] FIB activity Three tests were performed on 3 [illegible] days each in triplicate The values g[illegible] the tables are the means of the results from 1 experiment The lowest and the highest result obtained are given in parenthesis

The results are presen[illegible]d as per cent inhibition, if not otherwise stated i e

$$100 \quad \frac{\text{Release of label from control with serum} - \text{release of label from test}}{\text{Release of label from control with serum}}$$

TABLE 1 *Effect of Various Fractions of Fæces and some Carbohydrate Preparations on the Bactericidal Activity of Rat Serum on E coli For Further Details see Preparation of Extracts*

Material	Per cent inhibition of release of label*
Fæces, aqueous extract	84 (83 4 85 8)
Dialysable fraction	0 (–9 7, –6 5)
Non dialysable fraction	89 (88 1 89 5)
Fæces, non dialysable, non lipid fraction	
ETOH insoluble (= material HMW)	87 (87 2, 87 6)
ETOH soluble	60 (59 6, 60 4)
Extract of ordinary rat diet	
ETOH insoluble	30 (26 7, 33 0)
Gum tragacanth	0 (–4 1 –0 2)
Polysaccharide from *Papaver somniferum* L	0 (–3 4 –2 0)
Bovine glycoprotein, Cohn fraction VI	0 (–2 3, 1 0)

* The results are expressed as per cent inhibition of release of ^{32}P from labelled *E coli* The control with serum is taken as 0 (–1 3 1 2) per cent The control without serum gave 25 (21 5 30 1) per cent release of label

RESULTS

Localization in Fæces of Substances Exerting FIB Activity

Various fractions of fæces from germfree rats were tested for FIB activity (Table 1) An aqueous extract of fæces inhibited mark

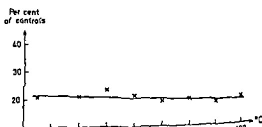


Fig 2 Release of ^{32}P from labelled *E coli* in media containing fæcal material HMW pretreated for 30 min in KRG at various temperatures Samples contained 20 mg lyophilized fæcal material HMW/2 ml KRG The control with serum is taken as 100 (96 0, 106 6) per cent release of ^{32}P The control without serum gave 14 (12 3, 15 1) per cent release of ^{32}P

TABLE 2 *Effect of Proteolysed Fæcal Material HMW on Serum Bactericidal Activity*

Material	Incubation time (hours)	Per cent inhibition of release of label		
		Dialysable fraction*	Non dialysable fraction	
			ETOH precipitated	ETOH soluble
Fæcal material HMW	17	12 (8.0, 18.5)	72 (71.0, 74.8)	0 (−10.7, −0.6)
Fæcal material HMW	192	13 (11.3, 14.3)	69 (67.2, 70.3)	48 (41.8, 52.3)
Fæcal material HMW − pronase	0		50 (41.7, 56.2)	2 (−2.0, 4.2)
Fæcal material HMW − pronase	192		49 (46.9, 49.7)	4 (−4.4, 10.9)
Pronase − fæcal material HMW	192	5 (3.2, 6.9)	0 (−15.9, −2.8)	0 (−26.4, −6.0)

* Standardization of the dialysable fractions 1 mg lyophilized material dissolved/ml KRG The dry weight of the dialysable fractions include salts from the incubation media, amino acids and peptides [illegible] of pronase and/or amino acids peptides and glycopeptides from proteolysed [illegible] cent inhibition of release of ^{32}P from labelled *E coli* [illegible]) per cent The control without serum gave 18 (15.5,

edly the bacteriolysis of normal rat serum on *E coli* FIB was non dialysable Extraction with chloroform methanol (4 1, by vol) and acetone did not diminish FIB activity of the resulting non lipid fraction The KRG soluble part of the lipid extract exerted no FIB activity The non lipid precipitate obtained after addition of ethanol (= material HMW) was a more potent inhibitor of serum bacteriolysis than the supernatant

Treatment by Heat of Fæcal Material HMW

Fæcal material HMW was incubated in KRG for 30 min at different temperatures As illustrated by Fig 2, FIB activity resisted heat treatment

Proteolysis of Fæcal Material HMW

Proteolysis for 17 hours provided a non-dialysable ethanol insoluble fraction exerting FIB activity while no activity was demonstrated in the non-dialysable ethanol soluble fraction (Table 2) After proteolysis for 192 hours however, FIB activity was demonstrated both in the ethanol insoluble and the ethanol soluble non dialysable fractions. Only negligible FIB activity could be demonstrated in the dialysable proteolyzed fractions

In order to investigate whether prolonged incubation at 37° C affected the factor, fæcal material HMW was tested for FIB activity before and after incubation at 37° C for 192 hours, without pronase FIB activity was of the same magnitude for both samples To elucidate whether the protease itself contributed to FIB activity, pronase alone was incubated for 192 hours at 37° C Neither the dialysable nor the non dialysable fraction inhibited serum bacteriolysis

Pretreatment of Fæcal Material HMW with Thiol blocking Agents (*N* ethylmaleimide or *p* chloromercuribenzoate) did not change FIB activity

Treatment of Fæcal Material HMW with a Quaternary Ammonium Compound (CTAB) gave 2 fractions, inhibiting serum bactericidal activity to about the same extent (Table 3)

TABLE 3 *Inhibition of Bactericidal Activity of Rat Serum on E coli Exerted by Fæcal Material HMW, Treated with CTAB (Fig 1)*

Fæcal material HMW	Per cent inhibition of release of label*
Treated with CTAB	
Precipitate B	83 (82.1, 83.7)
Precipitate F	76 (75.0, 77.3)
Unfractionated with CTAB	78 (76.9, 78.9)

* Results are given as per cent inhibition of release of ^{32}P from labelled *E coli* The control with serum is taken as 0 (−1.8, 2.1) per cent The control without serum gave 26 (24.7, 27.4) per cent release of label

Acid Hydrolysis

Paper chromatography of acid hydrolysates of fæcal material HMW illustrated the presence of sialic acids, galactosamine, glucosamine galactose, glucose, mannose, xylose, fucose, and arabinose (22) Hydrolysis of fæcal material HMW with an aqueous suspension of Dowex 50 (H^+) at 100° C for 1 hour provided, according to paper chromatographic examination of hydrolysates, a material free from sialic acids and less abundant in fucose (22) The partly hydrolysed and the original fæcal material HMW inhibited the release of ^{32}P from *E coli* by 74 (71 5, 78 3) and 61 (59 7, 61 5) per cent, respectively The control with serum was 0 (–2 1, 1 8) per cent inhibition The control without serum gave 19 (16 5, 21 6) per cent release of label

Localization in the Intestinal Tract of Substances Exerting FIB Activity

Fæcal material HMW exerted a stronger FIB activity than material HMW of contents of small intestine, caecum and colon (Table 4) Material HMW of the mucosal layer of caecum inhibited serum bacteriolysis more markedly than the mucosal layer of small intestine and colon As regards material HMW of the muscular/serosal layers, only a minor FIB activity could be demonstrated

TABLE 4 *FIB Activity of Material HMW of the Respective Intestinal Fractions*

Material HMW	Per cent inhibition of release of label*
Content of small intestine	68 (64 8 71 8)
Content of caecum	72 (69 3 74 0)
Content of colon	67 (65 9, 70 4)
Fæces	82 (76 9, 86 3)
Mucosal laye[illegible] of [illegible]mall intestine	24 (19 1, 29 8)
Mucosal layer of [illegible]um	67 (65 3 68 4)
Mucosal layer of [illegible]	35 (34 4, 36 9)
Muscular serosal [illegible] of small intestine	14 (10 4 16 8)
Muscular/ser[illegible]sal la[illegible]f caecum	27 (24 6 30 0)
Muscular/serosa[illegible] laye[illegible]s of colon	20 (17 2 22 0)

* The results are expressed as per cent inhibition of release of [illegible]P [illegible]m labelled *E coli* The control with serum is [illegible]k[illegible]n as 0 (–5 5 5 5) per cent The control with[illegible] serum gave 22 (18 5, 27 1) per cent release of [illegible]l

Yield of Material HMW from the Gut Segments of 4 Rats

As illustrated by Table 5, the content of caecum yielded about 80 per cent of the material HMW from the whole intestine

Investigation of the Specificity of the Test for FIB Activity

Extract of the ordinary rat diet was prepared according to the procedure used for fæcal material HMW The material HMW of ordinary rat diet inhibited serum bacteriolysis to some extent (Table 1) In order to investigate whether polar high molecular substances were able to inhibit serum bactericidal activity in general, aqueous solutions of 2 plant polysaccharides and 1 glycoprotein fraction were prepared FIB activity could not be demonstrated for gum tragacanth the polysaccharide fraction from *Papaver somniferum* L or for the bovine glycoprotein, Cohn fraction VI (Table 1)

DISCUSSION

Previous studies have shown that strangulation fluid from rats contains substances which inhibit the opsonic and bactericidal activity of rat serum on *E coli* (15, 17) In order to save valuable test material and to simplify technical procedures, estimation of humoral bactericidal activity was chosen as test method in the present study, using release of radioactivity into the medium *from* ^{32}P labelled *E coli* as a parameter of bactericidal *activity* (14, 16) Due to variations in bacterial *number, incorporation* of radioactivity as well as *serum activity* results have been compared only within the same experimental series

Recent studies have indicated that substances responsible for FIB activity may originate from the intestinal secretions of the animal (19) Fæces from germfree rats proved to be

TABLE 5 *Yield of Material (from 4 Rats), Collected after the Rats Had Been Fed a Low Molecular Weight Diet for 5 Days*

Source of material	mg material collected (wet weight)	mg material HMW (lyophilized)
Content of small intestine	1190	62
Content of caecum	35110	1300
Content of colon	2000	130
Mucosal layer of small intestine	2610	15
Mucosal layer of caecum	290	5
Mucosal layer of colon	220	2
Muscular/serosal layers of small intestine	5440	34
Muscular/serosal layers of caecum	3030	24
Muscular/serosal layers of colon	1370	37

a good source of FIB. Whenever an aqueous extract of faeces was dialysed, FIB was found to be localized to the non dialysable fraction only. Extraction of lipids produced an extract with no activity, while the non-extractable residue contained a substantial FIB activity. After precipitation with ethanol, FIB was recovered both in the supernatant and in the precipitate. The ethanol insoluble fraction was slightly coloured in contrast to the ethanol soluble material which was dark brown. The precipitate invariably proved to be a more potent inhibitor than the soluble material. Therefore, the precipitate was used for further studies. Demonstration of inhibitory substances both in the ethanol soluble and the ethanol insoluble fractions might be due to the distribution-equilibrium of the substances in question. A heterogeneous mixture of substances with inhibitory effect, however, cannot be excluded.

According to the fractionation procedure FIB should consist of polar substances of high molecular weight. It was not likely that FIB could be due to glycolipids since the lipid extracts exerted no FIB activity.

Neither heat treatment up to 100° C for 30 min nor SH blocking agents abolished FIB activity. Furthermore, proteolysis for 8 days did not markedly reduce FIB activity of the non-dialysable, ethanol insoluble fraction. These findings indicated that FIB activity was not due to substances of pure protein nature. It could be argued that proteolysis might be repressed by substances in the incubation mixture. If so, this was of minor importance, since the presence of ninhydrin reactive components was demonstrated in the dialysable fraction already after proteolysis for 17 hours. As FIB activity was intact after extensive digestion with pronase, it might be due to substances of polysaccharide or glycoprotein nature. Glycoproteins with a considerable carbohydrate portion, such as the mucins, are notoriously resistant to proteolytic digestion (10).

Treatment of faecal material HMW with a quaternary ammonium compound (CTAB) resulted in 2 fractions of approximately similar FIB activity. As CTAB precipitates polyanions, these observations might indicate that weakly acidic molecules or molecules with different pI are responsible for FIB activity.

Weak acid hydrolysis of faecal material HMW invariably provided more pronounced FIB activity than the unhydrolysed material. This might be due to degradation of substances in faeces enhancing the bactericidal activity of serum or truly enhanced FIB activity following weak acid hydrolysis. Such hydrolysis resulted in release of sialic acids and fucose. Both these monosaccharides are usually found in glycoproteins.

The results of the present experiments indicate that substances responsible for FIB activity of faecal material are of polysaccharide and/or glycoprotein nature. Previous studies indicated that mucin preparations and polysaccharides of different origin enhanced the lethal effect of bacteria on mice if injected

together with bacteria into the peritoneal cavity, as reviewed by *Olitzki* (11) Polysaccharides and glycoproteins did not generally exert FIB activity in our test system, since no inhibitory activity could be demonstrated in the case of gum tragacanth, the polysaccharide fraction from *Papaver somniferum* L, or in the case of the bovine serum glycoprotein fraction tested

The origin of substances causing FIB activity was also studied The pelleted diet caused moderate FIB activity To eliminate this factor, the rats were fed a chemically defined diet which left no material after the employed extraction procedure Fæcal material collected from the 2nd to the 5th day exerted the same FIB activity as the fæcal material collected from the 5th to the 10th day Therefore, the shorter period was chosen for feeding the rats the special diet

Contents from all intestinal segments tested produced substantial FIB activity Whenever material HMW of the mucosal layers as well as the muscular/serosal layers of the respective segments were tested, the results indicated that substances exerting FIB activity could be secreted from the mucosal layer of the whole intestinal tract, especially from that of caecum FIB activity would be expected to be higher in the secretions than in the mucosa, due to a higher concentration of secretions in the intestinal contents than in the mucosal cells

Previous studies have shown that FIB activity caused by strangulation fluid from germfree rats was due to non dialysable, high molecular substances which also resisted heat treatment up to 100° C for 30 min (19) It seems reasonable to assume that the inhibitory substances in strangulation fluid might be identical with inhibitory substances studied in the present experiments

The intestinal flora is a mixture of many different types of micro organisms The micro-organisms may act upon each other as well as being subjected to the influence of dietary and secretory factors The present results indicate that a host factor, capable of inhibiting humoral bactericidal activity on *E coli*, may be secreted into the intestinal lumen Therefore, FIB may be of importance not only for the outcome of intestinal strangulation obstruction, but also for the establishment of the normal intestinal flora

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CASEIN INDUCED AMYLOIDOSIS IN T-CELL DEPRIVED MICE

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The degree of casein induced amyloid development was found to be similar in both untreated and adult thymectomized irradiated bone marrow grafted CBA mice Less amyloidosis occurred as a result of either adult thymectomy alone or lethal irradiation and bone marrow grafting of non thymectomized animals Circulating antibodies to casein were found in similar concentrations in all four situations It appears that amyloid development is not directly affected by the thymus or the T lymphocyte

Only the spleen reticuloendothelial or reticular cells (*Teilum* 1956, *Cohen* 1960, *Ranlov* & *Wanstrup* 1967) are known to be involved in amyloid formation The importance of the thymic influence and/or T-lymphocyte is still not well-defined

The purpose of the present study was to reinvestigate the influence, if any, of the thymus and the circulating T-cell pool in amyloidogenesis using a combination of adult thymectomy and irradiation to deplete the T cell pool (*Doenhoff et al* 1970) Partial reconstitution of the T-cell pool following irradiation can be achieved by leaving the thymus intact (*Davies et al* 1971) and the adult thymectomy alone may be considered to remove any humoral influence emanating from the thymus while preserving the T-cell pool intact (*Doenhoff et al* 1970)

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MATERIALS AND METHODS

CBA/Lac male mice were used throughout and were divided into four groups treated as follows

1) Untreated controls 2) Adult thymectomy at 8 weeks 3) Non thymectomized mice given 850 r irradiation and an intravenous injection of 5×10^{6} syngeneic bone marrow cells (T cell reconstituted mice) 4) Adult thymectomized animals treated as in group 3 (T cell deprived mice) The techniques for thymectomy, irradiation and bone marrow grafting have been described previously (*Davies et al* 1966) and the effectiveness of thymectomy was examined macro- and microscopically at autopsy

Casein was first administered approximately 8 weeks after the operations 0 5 ml of a 5 per cent casein solution in 0 25 per cent sodium hydroxide being injected subcutaneously 6 times a week for 7 weeks Some mice in each group were treated with saline (controls)

Two days after the last casein inoculation the animals were killed Lung, liver kidney, spleen peripheral lymph nodes, thymus small intestine cor, caecum and thyroid gland were taken for histology and stained with periodic acid Schiff (PAS) and alkaline Congo red

Amyloid was identified by its morphology and by its birefringence with Congo red under crossed polars

The degree of spleen amyloidosis was calculated on PAS stained sections and graded from 1 to 6 (three indicating an unbroken ring of amyloid

around spleen follicles) (*Christensen & Hjort* 1959)

The presence of *circulating anti casein antibodies* was tested in a single-radial diffusion method described previously (*Ebbesen* 1971) Wells in gels containing 2 per cent agarose and 1 per cent casein were filled with serum obtained at autopsy and pooled for each group

RESULTS

Amyloid if present, was always found in the spleen Grade 4 spleen amyloidosis was accompanied by liver amyloidosis No amyloid was observed in other organs

Spleen amyloid developed with nearly the same frequency in untreated and in T cell deprived animals (*Table 1*) Significantly fewer adult thymectomized mice and T-cell reconstituted mice developed amyloid than non-operated mice No amyloid was present in mice not given casein

Neither leucocyte count, nor granulocyte percentages nor haematocrit values or antibodies to casein correlated with amyloid development

The mice looked healthy throughout the experiment although four died during caseination

DISCUSSION

Conflicting results have been obtained concerning the effect of the thymus and the T-cell on casein induced amyloid formation Adult thymectomy alone or in combination with irradiation before caseination were found by *Ranloz* (1966) to accelerate amyloidosis, yet he found that antilymphocyte serum, if administered to adult mice during caseination reduced amyloid formation (*Ranlov* 1967) Neonatal thymectomy has been shown to have either no effect (*Rohde* 1965, *Clerici et al* 1966) or an enhancing effect (*Ebbesen* 1971) The development of casein induced amyloidosis in mice with congenital aplasia of the thymus has recently been reported to occur after an induction time shorter than that in normal litter mates (*Hardt & Claesson* 1972)

In the present results, deposition of amyloid in the spleen is not increased following adult thymectomy and irradiation and appears to be inhibited either after irradiation or thymectomy alone, indicating that although the thymus and/or the T cell pool may be in some way involved, they do not have a direct influence on amyloid pathogenesis

TABLE 1 *Spleen Amyloid Leucocyte Count and Casein Antibody in Caseinated CBA Mice*

Pretreatment		No of mice	Number with amyloid	Spleen P	Spleen Range amyloid	Spleen Mean amyloid	Leucocyte count	Per cent granulocytes	Casein antibodies Radial diffusion diameter in mm
None	casein	20	12		0-4	2.2	3838 ± 1207	41 ± 12	3.8
	control	4	0				1410 ± 543	34 ± 36	
Thymectomy	casein	21	6	< 0.02	0-4	0.9	3459 ± 1393	31 ± 8	3.7
	control	4	0				1950 ± 916	35 ± 7	
Irradiation BM cells (T-cell reconstituted)	casein	15	2	< 0.01	0-3	0.4	6585 ± 1552	42 ± 12	3.6
	control	3	0				4187 ± 3493	27 ± 11	
Thymectomy + irradiation BM cells (T cell deprived)	casein	20	11	> 0.1	0-4	1.7	4522 ± 364	50 ± 13	3.9
	control	3	0				2947 ± 1579	25 ± 8	

If thymus cells are involved in amyloid formation, an indirect influence via B lymphocytes (*Dennert* & *Lennox* 1972) seems probable Transfer experiments also seem to discredit T-lymphocytes as the more important cell Grafting of T-lymphocytes from syngeneic donors sensitized with casein to mice in caseination does not accelerate amyloid formation (*Clerici et al* 1969, *Hardt* 1971), whereas transfer of sensitized spleen cells (*Hardt* 1971) and transfer of unsensitized lymph node cells (*Hardt et al* 1972) have some enhancing effect We therefore favour the hypothesis (*Ebbesen* 1971) that amyloid fibrils are produced in antibody producing lymph node cells (*Ebbesen et al* 1969) and later stored in spleen macrophages

Occurrence of antibodies to casein in all groups may imply that the effect on humoral immunity to this antigen is largely thymus independent

It is possible that an investigation of the part played by the B lymphocyte in amyloid formation will prove enlightening

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THE ORIGIN OF IMMUNOGLOBULIN-G IN BOVINE TEARS

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Studies with radio-iodinated albumin IgG 1 and IgG 2 in calves revealed that IgG 1, when compared with albumin and IgG 2, is selectively transferred from blood to tears The majority of IgG 1 in tears seemed to be of plasma origin The transfer rate of IgG 2 from blood to tears was slightly below that of albumin During development of experimental keratoconjunctivitis produced by infection with *Moraxella bovis* the transfer rate of IgG 1 IgG 2 and albumin was increased and their concentration ratios approached the serum pattern The selective transfer of IgG 1 from blood to external secretions may account for the higher catabolic rate of IgG 1 when compared with IgG 2 in the bovine species.

Quantitative determination of immunoglobulins in bovine tears has revealed that the concentration of IgG 1 is consistently higher than that of IgG-2 and albumin (5, 6, 14) The finding that the IgG 1 albumin ratio is several times higher in tears than in serum (14) indicates that IgG 1 is selectively transported from plasma, locally synthesized, or a combination of these possibilities

The aim of the present investigation was to study the origin of IgG-1, IgG 2 and albumin in tears and to examine the possibility of a selective transport of IgG 1 from plasma

MATERIALS AND METHODS

The experiments were performed with radiolabelled IgG 1, IgG-2 and albumin The experimental model with radiolabelled proteins was as described by *Strober et al* (16) in their study of the origin of salivary IgA in man

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Experimental Animals

Four calves of the Red Danish Milkbreed 4 to 10 months old were used (Table 1) Experimental keratoconjunctivitis was produced by infection with *Moraxella bovis* as previously described (13, 15) In each calf one eye was selectively irradiated with ultraviolet (UV) light (300 watt) for 10 minutes at a distance of 60 cm on the day before, and again immediately prior to the inoculation The other eye served as a control

Isolation and Radio iodination of Proteins

IgG 1 and IgG 2 were isolated from serum by DEAE Sephadex A 50 ion exchange chromatography (10) IgG 1 was obtained from two IgG 2 deficient sera IgG 2 was isolated as the fall through fraction from the serum of a hyperimmunoglobulinaemic cow The immunoglobulin preparations were rechromatographed and only the ascending parts of the protein peaks were collected and concentrated The IgG 1 and IgG 2 preparations were further purified by Sephadex G-200 gel filtration In one experiment (J No 462/70) IgG 1 was isolated from colostrum (1) All immunoglobulin preparations were immunoelectrophoretically pure Albumin used for labelling was a salt precipitated commercial preparation (Serum-Albumin vom Rind trocken, reinst (Behringwerke AG))

The proteins were labelled with carrier free ^{125}I and ^{131}I by the iodine monochloride method of

McFarlane (8) Non protein bound iodine was removed by chromatography on a resin column (Amberlite Resin IRA-400) More than 99 per cent of the radioactivity in the final solution was precipitable with 10 per cent trichloroacetic acid (TCA) In the immunoglobulin preparations less than 1 atom and in the albumin preparations less than 2 atoms of iodine were, on the average, incorporated per molecule DEAE Sephadex chromatography and Sephadex G 200 gel filtration (14) of the labelled preparations, mixed with a normal serum pool, revealed that the radio iodinated proteins were homogeneous and had retained their typical elution positions To prevent denaturation due to self radiation, the preparations were diluted with bovine serum albumin to a specific activity less than 2 μCi/mg protein The preparations were injected immediately after the labelling procedure, or after storage overnight at 4° C

Administration of Labelled Proteins

Two proteins, labelled with ^{125}I and ^{131}I, respectively, were simultaneously injected through a plastic catheter into the jugular vein The amount of radioactivity in each preparation ranged from 560–4 050 μCi This high dose was necessary to determine the small fraction of protein bound radioactivity in tears (see below) After 15 minutes a blood sample was drawn for the estimation of plasma volume subsequent samples were taken daily at the same hour for 3 weeks The proportion of non bound radioactivity in plasma after TCA precipitation ranged from 0 3 to 1 4 per cent (average 0 7 per cent) of the total plasma radio activity Lugol s iodine solution was administrated to impede thyroid uptake of radio iodide (10)

Collection of Materials

Tears were obtained from the lower conjunctival sac by means of a 1 000 μl constriction pipette Approximately 5 ml were obtained from each eye The flow rate was tentatively defined as the volume of tears collected per minute The tear samples were centrifuged at 1 500 G for 10 minutes and filtered through a Millipore filter with a pore size of 220 nm The first tear sample was collected on the 4th day after the administration of labelled proteins (immediately prior to the first UV irradiation) and the second on the 5th day (immediately prior to the second irradiation and the subsequent inoculation of *M bovis*) Calf J No 462/70 was inoculated 2 days after the injection of labelled proteins and the first tear sample was obtained 4 days after the injection Subsequent samples were collected until the protein bound activity in tears had dropped below the limit of quantitation

Counting Procedure

Radioactivity was measured in a thallium activated NaI scintillation well counter equipped with a single channel discriminator to differentiate ^{125}I and ^{131}I activity Plasma and corresponding tear samples were counted in 4 ml aliquots immediately after each sampling procedure The protein bound radioactivity was determined as the difference between the activity in the sample and the activity in the supernatant after precipitation with TCA The determination of the small amount of protein bound radioactivity in tears (see below) was relatively imprecise since it was derived from the difference between two high values The very low non protein bound radioactivity in plasma justified that calculations were based upon the total plasma activity At the completion of each experiment the series of plasma samples from each calf were counted together with standard solutions (1 2 000 dilutions of the injected preparations)

Quantitation of Proteins

Immunochemical quantitation of albumin IgG 1 and IgG 2 was performed by single radial immunodiffusion (7, 14) Monospecific antisera were prepared as previously described (10, 14) The anti bovine IgG 2 was not directed against the entire IgG 2 antigenic spectrum (10 14) Total protein in serum and tears was determined by a micro-Kjeldahl method using 6 25 as a convers on factor

Calculations

The plasma radioactivity curve was plotted versus time on semilogarithmic paper and analysed by the method of *Nosslin* (12)

The specific activity of proteins in tears and serum was calculated as protein bound counts per minute per mg protein (cpm/mg protein) If the specific activity of a certain protein is equal in tears and serum at the same time the tear protein must be derived solely from the blood either by a passive or a selective process On the other hand if the specific activity in tears is lower than that in the corresponding serum sample local synthesis of unlabelled protein must occur somewhere between the plasma compartment and the tears

The excretion of IgG 1 and IgG 2 was studied in relation to the excretion of albumin In one experiment IgG 1 and IgG 2 were studied simultaneously in the same animal (J No 181/71)

An IgG albumin tear activity ratio above the same ratio in plasma was considered indicative of a selective external transfer of IgG

TABLE 1 *Age, Weight and Metabolic Data of 4 Calves*

Calf No	Age (months)	Weight (kg)	Radio-iodine labelled protein	Plasma volume* (ml/kg)	Fractional catabolic rate§ (%/day)	Half life time† (days)	Distribution ratio$ (% intravasc.)	Transcapillary escape rate" (%/day)
62/70	4	93° (110)ɔ	Albumin	47 4	8 6	17 9	45	108
			IgG 1	51 9	4 8	26 4	55	76
83/71	4	92 (108)	Albumin	62 1	7 2	19 5	49	109
			IgG 1	53 8	5 7	19 7	62	52
82/71	7	246 (255)	Albumin	43 9	7 4	21 6	43	58
			IgG 2	37 8	5 9	22 3	52	33
181/71	10	286 (291)	IgG 1	41 5	6 2	17 9	63	36
			IgG 2	43 6	4 1	26 9	63	40

* Plasma volume *Total activity injected/activity in 1 ml plasma at zero time (the 15 minutes sample representing 98 per cent of injected dose)*
§ Fractional catabolic rate Fraction of the intra vascular protein pool catabolized per day
† Half life time The true half life of the labelled protein.
$ Distribution ratio The ratio between the total extra vascular pools and the intra vascular pool
" Transcapillary escape rate (total exchange rate) The fraction of the intra vascular pool entering the extra vascular pools per day
° Weight at the day of injection
ɔ Weight 3 weeks after the injection

RESULTS

The metabolic parameters of the studied proteins are given in Table 1 The fractional catabolic rate of IgG-1 in the calves was lower than that normally reported for adult animals (10), and the lowest figure was found in the youngest calves When IgG-1 and *IgG 2 were studied simultaneously (J No* 181/71), the fractional catabolic rate of IgG 1 was higher than that of IgG 2, in accordance with findings in adult cattle (10) The distribution between intra- and extra-vascular compartments differed for IgG and albumin Thus 55-63 per cent of IgG and 43-49 per cent of albumin were located intravascularly A similar differential distribution has been found in other mammals (17)

Normal Eyes

After intravenous injection of radioiodinated proteins a surprisingly small amount of the total radioactivity in tears was bound to protein In normal eyes the average percentage of protein-bound activity of albumin, IgG-1 and IgG 2 was $8 0 \pm 0 8$ ($n = 13$), $28 4 \pm 2 4$ ($n = 12$) and $7 9 \pm 1 5$ ($n = 13$), respectively, (mean ± s e m) In order to examine whether this might be due to degradation and de iodination small aliquots of each labelled protein were mixed with tears and saline respectively, to give a radioactivity *similar to that of tear samples during* the excretion experiments Seven samples were taken during the next 24 hours of incubation at 37° C and precipitated with TCA Counting revealed no rise in the non-precipitable radioactivity and no difference between tear and saline samples Addition of trace amounts of sodium iodide (^{131}I) to freshly secreted tears and subsequent incubation at 37° C for 30 min indicated that no TCA precipitable, or non-dialysable radioactivity was *formed After intravenous injection* of sodium iodide (^{131}I) in three calves, the total radioactivity in tear and plasma samples was non precipitable with TCA (A detailed description of the excretion of iodide will be given elsewhere)

The absolute levels of IgG-1 and albumin

TABLE 2 *Levels (mg/100 ml) of Total Protein, IgG-1 and Albumin in Serum and Tears*

Time (days)	Serum				Tears from normal eye					Tears from diseased eye* §				
	Total pro-tein	Albu-min	IgG-1	Ratio IgG 1 / Alb	Total pro tein	Albu-min	IgG-1	Ratio IgG 1 / Alb	"Flow rate" (ml/min)	Total pro tein	Albu min	IgG 1	Ratio IgG 1 / Alb	"Flow rate (ml/min)
–1	5570	2650	1200	0 45	340	1 6	3 4	2 1	0 2	410	1 2	4 9	4 1	0 2
0	5620	2640	1160	0 44	250	1 6	4 7	3 0	0 1	140	13 0	8 1	0 62	0 1
3	5510	2690	1180	0 44	340	1 6	6 5	4 0	0 1	10	2 3	2 4	1 0	0 2
Mean	5567	2660	1180	0 44	310	1 6	4 9	3 0						
2	5880	2540	1580	0 62	580	2 3	7 5	3 3	0 2	150	5 4	2 8	0 52	0 2
3	5740	2590	1420	0 55	540	1 4	3 5	2 5	0 1	70	6 2	4 2	0 68	0 3
4	6030	2680	1520	0 57	430	1 2	3 9	3 3	0 2	180	6 7	6 6	0 99	0 2
Mean	5883	2603	1507	0 58	517	1 6	5 0	3 0						

* *J No 183/71* One eye was inoculated by conjunctival instillation of 0 2 ml horse serum broth culture of *M bovis* at day 0 One day before (–1) and immediately prior to inoculation this eye had been UV irradiated On day of inoculation (0) skin hyperaemia was present around the eye and there was increased moisture in the conjunctival sac On day 3 superficial keratitis, conjunctivitis and lachrymation were observed

§ *J No 462/70* UV-irradiation and inoculation procedure were as described above Severe keratitis conjunctivitis and lachrymation were present on day 2 On day 3 initiation of corneal vascularization was observed On day 4 conjunctival inflammation had decreased

TABLF 3 *Levels (mg/100 ml) of Total Protein, IgG 1 and Albumin in Serum and Tears*

Time (days)	Serum					Tears from normal eye					Tears from diseased eye*				
	Total pro tein	Albu min	IgG 1	IgG 2	Ratio IgG 1 / Alb	Total pro tein	Albu min	IgG 1	Ratio IgG-1 / Alb	'Flow rate" (ml/min)	Total pro tein	Albu min	IgG 1	Ratio IgG 1 / Alb	'Flow rate (ml/min)
1	6310	3110	1510	43	0 49	510	1 8	3 1	1 7	0 5	460§	1 6	3 4	2 1	0 5
0	6070	3000	1450	31	0 48	370	2 6	4 4	1 7	0 2	180	1 4	2 2	1 6	0 8
1	6410	3050	1480	36	0 49	370	2 2	5 2	2 4	0 2	150	4 0	4 3	1 1	0 5
3	6670	3300	1600	38	0 48	400†	2 3	5 3	2 3	0 2	120	2 5	4 7	1 9	0 6
4	6420	3100	1500	21	0 48	400	2 4	5 3	2 2	0 3	150	3 0	4 3	1 4	0 8
6	6370	2420	1540	34	0 64	460†	2 7	5 3	2 0	0 2	130	1 4	3 3	2 4	0 8
8	6180	3050	1510	39	0 50	470†	2 9	5 0	1 7	0 2	150	1 5	3 6	2 4	0 6
Mean	6347	3004	1513	35	0 51	426	2 4	4 8	2 0						

* J No 182/71 UV irradiation and inoculation procedure was as described in legend (*) to *Table 2* The day after inoculation (1) there was marked conjunctivitis and serous lachrymation On day 3 severe keratitis moderate conjunctivitis and lachrymation were observed Beginning circum-corneally vascularization and decreasing conjunctival inflammation were present on day 4 *Thereafter, corneal* vascularization processed and healing took place from the periphery

§ Slight hyperaemia of conjunctival mucosa

† The calf was rather sturbed during sampling of tears from the normal eye and restraint was difficult

TABLE 4 *Levels (mg/100 ml) of Total Protein, IgG 1 and Albumin in Serum and Tears*

Time (days)	Serum				Tears from normal eye					Tears from diseased eye*				
	Total protein	Albumin	IgG 1	Ratio IgG 1 Alb	Total protein	Albumin	IgG 1	Ratio IgG 1 Alb	Flow rate (ml/min)	Total protein	Albumin	IgG-1	Ratio IgG 1 Alb	Flow rate (ml/min)
-1	6900	3120	1400	0.45	440	0.7	3.7	5.3	0.4	520	0.6	3.4	5.7	0.4
0	7050	3230	1400	0.43	340	0.6	3.7	6.2	0.3	180	105.0	49.0	0.5	0.4
1	6930	3150	1380	0.44	380	1.1	6.2	5.6	0.3	200	7.0	6.3	0.9	0.7
2	6770	3180	1350	0.42	350	1.2	6.8	5.7	0.3	290	10.0	8.4	0.8	0.3
3	6450	3260	1350	0.41	430§	2.6	8.4	3.2	0.3	320	2.2	6.4	2.9	0.3
4	6670	3150	1360	0.43	530§	2.3	5.7	2.5	0.2	350	1.2	7.1	5.9	0.5
7	7010	3080	1340	0.44	500	1.1	7.2	6.5	0.3	230	0.9	5.7	6.3	0.6
12	7220	3150	1410	0.45	450§	2.7	7.8	2.9	0.2	230	2.0	7.0	3.5	0.4
17	6680	3280	1370	0.42	420	2.0	5.6	2.8	0.2	320	1.0	5.9	5.9	0.3
Mean	6853	3178	1373	0.43	405	1.1	5.5	5.4						

* J No 181/71 UV irradiation and inoculation procedure were as described in legend (*) to Table 2. The day after inoculation (1) the conjunctival mucosa was hyperaemic and there was marked serous lachrymation. The conjunctival inflammation decreased during the next days (2, 3 and 4). On day 7 there was severe keratitis. Progressing corneal vascularization was observed on day 12, conjunctiva was of normal appearence. On day 17 the vessels had nearly reached the centre of cornea.

§ Traumatic damage of conjunctiva during sampling of tears (results not included in the mean)

in tears and serum are shown in Tables 2, 3 and 4. The average IgG-1 albumin ratio in tears varied from 2.0 to 5.4. In contrast the IgG 1 albumin ratio in serum was approximately 0.5.

The specific activity of IgG 1 in tears tended to be lower than that in the corresponding serum samples which had an average ratio of 0.88 ± 0.08 $(n = 12)$.

The ratio between the radioactivity bound to IgG-1 and albumin in normal tears was on the average 5.0 times higher than that in plasma at the same time (Table 5).

The level of IgG 2 in tears was too low to be determined by the single radial immunodiffusion technique (i.e. < 0.6 mg/100 ml see refs 10 and 11). The experiments with radiolabelled IgG 2 and albumin revealed that the IgG 2 albumin ratio in tears was about 20 per cent lower than the same ratio in plasma (Table 6).

The ratio between the IgG 1 and IgG 2 bound activity was on the average 7.5 times higher in tears than in plasma (Table 7).

The specific activity of albumin was somewhat higher in tears than in serum with an average ratio of 1.24 ± 0.06 $(n = 13)$. The ratio between the albumin bound radioactivity in tears and plasma remained fairly constant being independent of variations in the plasma radioactivity.

Diseased Eyes

All eyes exposed to *Moraxella bovis* and irradiation with UV-light developed kerato

TABLE 5 *Ratio between the Protein bound Radioactivity of IgG 1 and Albumin in Plasma and Tears*

Time (days)	Plasma	Tears from normal eye	Tears from diseased eye* §
-1	1.2	3.4	3.0
0	1.1	4.6	1.4
3	0.8	3.8	2.1
2	4.5	27.7	11.5
3	4.9	38.9	12.1
4	5.6	23.3	13.8

* § See legends to Table 2

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ANALYSIS OF THE VARIATION IN LYMPHOCYTE RESPONSE TO PHA IN NORMAL SUBJECTS

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Among normal persons a marked variation in DNA synthesis is observed in lymphocyte culture stimulated with PHA We have studied some of the factors that may be responsible for this normal variation Liquid scintillation counting of the uptake of tritiated thymidine in cultured cells stimulated by PHA was taken as a measure of the DNA synthesis 1 A study of 53 normal men and 42 normal women showed that the transformation was correlated to age, but not to sex 2 Six normal subjects were followed from four to twelve months Although the lymphocyte transformation varied with time, the level of transformation remained constant 3 A study of the transformation in seven pairs of monozygous twins and in five pairs of dizygous twins failed to show a genetic influence 4 Finally autoradiography and liquid scintillation counting were carried out on the same cultures It appeared that the transformation as measured by liquid scintillation is propartional to the number of cells synthesizing DNA

Nowell (13) showed that the addition of phytohaemagglutinin (PHA) to cultures of lymphocytes from peripheral blood induced a number of changes in these cells The changes mainly consist of DNA synthesis, mitosis and a blast like transformation of the cells Accordingly, the incorporation of tritiated thymidine, studied either by autoradigraphy or liquid scintillation counting (*Baserga & Kisieliski* (1) *Caron et al* (2)), the mitotic index or the morphological changes of the cells (*Nowell* (13), *Cooling & Quaglino* (4)) have been used to quantitate the lymphocyte response to PHA

The lymphocyte response is generally believed to be associated with the delayed hypersensitivity phenomenon and has thus been used to study the immunological status of patients with many different diseases. But also among normal persons a large variation is found The present study was carried out in order to analyse some of the factors that might be responsible for this normal variation

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MATERIALS

The persons studied were members of the staff non hospitalized relatives, or healthy patients who remained in hospital on social indications exclusively In subjects aged 70 years or more, a normal sedimentation rate haemoglobin concentration, granulocyte and lymphocyte counts and urinary sediment were considered the prerequisites for inclusion in the study Subjects who were taking medicaments permanently or during the last 48 hours were excluded

The venous punctures on fasting subjects were all made between 7 30 and 9 00 a m From each blood sample, three or four parallel PHA-stimulated cultures and one unstimulated were established If not otherwise stated each person was studied on two or more occasions. As an expression of the ability of the lymphocytes from a particular person to respond to PHA *in vitro*, the average of all determinations were used

METHODS

Preparation and Culture of Lymphocytes

Venous blood was defibrinated by shaking with glassbeads for 10 minutes One third volume of a 3 per cent gelatine solution (M W 190000) was added to the serum and the cells were allowed to sediment (*Coulson & Chalmers* (3)) The lymphocyte rich supernatant was resuspended twice in Hank s solution and centrifuged for 10 minutes at 300 G at room temperature The cells were finally suspended in McCoy's medium supplemented by 30 per cent foetal calf serum, penicillin 100 iu/ml, and streptomycin 0 1 mg/ml The mononucleated white cells were counted in a Burker Turk Counting chamber The lymphocyte recovery was approximately 50 per cent The granulocyte contamination was less than 2 per cent Aliquots of 2 ml (containing 2×10^6 lymphocytes) were cultured for 46 hours in a mixture of 95 per cent air and 5 per cent CO_2 at 37° C Phytohaemagglutinin (Burroughs Wellcome) in a concentration of 100 µg/ml was added (*Fit gerald* (5))

Determination of ³H Thymidine Incorporation

With a view to measuring the DNA synthesis, ³H thymidine (Amersham 0 5 µCi/ml culture) was added to each culture three hours before harvest After 46 hours the cells were denaturated and fixed on Millipore filters according to *Mosedale & Parke* (12) The incorporation of ³H thymidine in DNA was measured in a liquid scintillation system (*Sorensen et al* (24)) The results were expressed as counts per minute (cpm) per ml of culture after subtraction of the value of the unstimulated culture The values of the unstimulated cultures were always less than 5 per cent of the corresponding stimulated cultures

Autoradiography

Approximately 0 5 ml of the cell suspension was centrifuged and the supernatant removed The cells

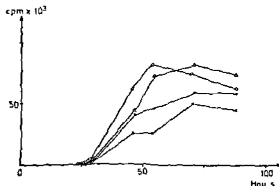


Fig 1 ³H thymidine uptake in PHA stimulated lymphocytes from four persons measured after 24 32 46, 52, 72 and 92 hours

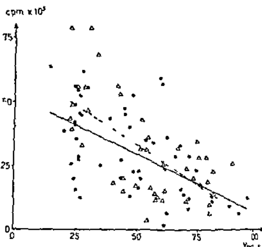


Fig 2 ³H thymidine uptake in PHA stimulated lymphocytes from 42 women (△) and 53 men (O) correlated to age The regression lines for men (—) and women (– – –) are shown

were resuspended twice in a mixture of one part glacial acetic acid and absolute methanol One small drop of the suspension was blown out into a wet and clean slide which was air dried The cells were stained in a 2 per cent solution of orcein in 50 per cent acetic acid for 15 minutes The slides were dipped into Ilford nuclear emulsion K2 and exposed for four days at 4° C After development in amidol the slides were mounted in DePex The black silver grains were seen very clearly against the pale brown cell nuclei The background was negligible A 40 x objective was used for the counting and all cell nuclei covered by grains were considered positive

Comments

The argument why an incubation time of 46 hours is used emerges from Fig 1 Four different persons were studied and the lymphocyte transformation was measured as described above The cultures were terminated after 24 32 46 52, 72 and 92 hours It appears that the uptake is steadily increasing during the interval from 30–50 hours Subsequently the incorporation tends to reach a plateau This seems to reflect that the cells enter the G phase mitosis or even a G of a new cell cyclus We feel that the period 30 50 hours mainly covers the S period of the cells and that differences in thymidine uptake during this period reflect the differences in lymphocyte response to PHA

RESULTS

Age and Sex

Forty two women and 53 men were studied Their ages ranged from 14 to 97 years In

TABLE 1 *^3H Thymidine Uptake in Lymphocytes from Six Persons The Interday—and Day to Day Variations are Calculated*

Name and age	Day	Mean cpm × 10³	S D × 10³	Coefficient of variat	Mean of all cpm × 10³	S.D × 10³	Coefficient of variat
MR 27	0	79 2	8 7	11 0			
	14	82 8	10 9	13 2	64 7	15 8	24 4
	21	59 5	13 5	22 7			
	35	56 3	10 5	19 7			
	46	45 8	4 6	10 0			
CL 42	0	59 8	4 3	7 2			
	15	45 5	2 0	4 4	59 0	10 1	17 1
	46	60 7	2 6	4 3			
	60	70 0	8 5	11 0			
AS 45	0	43 0	3 6	8 4			
	7	77 2	7 2	9 3	53 2	17 5	32 3
	56	54 6	10 2	18 7			
	84	37 8	9 0	23 8			
AM 47	0	12 9	0 5	3 9			
	14	18 2	0 01	0 1			
	21	24 4	0 6	2 5			
	156	12 6	0 7	5 6	20 1	8 8	43 8
	163	37 9	10 9	29 8			
	170	16 3	3 0	18 4			
	197	18 1	4 0	22 0			
DK 26	0	63 9	4 0	7 8			
	7	63 3	7 3	11 5			
	14	44 2	1 4	3 2	59 1	11 4	19 3
	284	73 1	3 1	4 2			
	339	51 1	5 6	11 0			
HS 24	0	62 1	7 9	12 7			
	7	55 2	1 5	2 7	56 0	7 3	13 0
	36	60 8	4 6	7 6			
	64	46 0	5 5	12 0			

Fig 2 the average values from each person have been plotted against age The results obtained in men and women have been treated separately A correlation coefficient of −0 56 was found in men and −0 65 in women These coefficients differed highly significantly from zero ($p < 0.001$), indicating that the ability of lymphocytes to respond to PHA *in vitro* decreases with advancing age of the individual Furthermore, the regression lines were calculated In men it was Y (cmp × 10³) = 52 42 −0 44 X (age in years) In women it was Y = 64 07 −0 62 X The regression coefficients (−0 44 and −0 62) differed both significantly from zero ($p < 0.001$), but there was no difference between them

Daily Variation

Experiments were carried out in order to elucidate the following

1 Is the variation in PHA response on two or more occasions different from the observed variation between parallel cultures

2 How much does the PHA response vary in the one and the same person if cultures are studied on a number of occasions over a period of several months

Six normal persons were studied four to seven times during a period of two to twelve months (Table 1) On each occasion three parallel cultures were established An analysis of variance showed that the between days

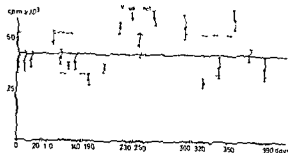


Fig 3 ³H thymidine uptake in PHA stimulated lymphocytes from one person (L K) followed for 390 days Each point represents the mean and standard deviation of triplicate cultures The mean (——) and S D (-----) are shown for the whole period (n = 18, mean = 42 6 × 10³ cpm, S D = ± 10 1 × 10³ cpm) The horizontal lines represent the mean and S D of all determinations.

variation is larger than the within-day variation ($p < 0.01$)

One person (L K) was followed for 390 days (Fig 3) During this period he suffered from a viral pneumonia, but otherwise his health was excellent The figure shows that the level of thymidine uptake remains fairly constant during the period studied The same conclusion may be drawn from Table 1 concerning the levels in the six normal persons

Genetic Influence

In order to see whether a genetic component might be responsible for the variation in lymphocyte response to PHA, a study of twins was carried out

Seven pairs of healthy male monozygous twins and 5 pairs of healthy male dizygous twins were studied, Zygosity had been proven by examination of thirteen groups of blood, serum and enzyme

The condition under which the sampling was carried out was the same as in the remainder of the study, except that determination of the lymphocyte response in the majority of cases was done once only

The results are seen in Table 2 The intrapair variance was calculated by squaring the differences between the partners in the individual pairs of twins The final mean intrapair variance in the two series (monozygous and dizygous) was calculated as the sum of

TABLE 2 ³H *Thymidine Uptake in Lymphocytes from Seven Pairs of Monozygote Twins and Five Pairs of Dizygote Twins*

MZ twins			DZ twins		
Pair no	Age	cpm	Pair no	Age	cpm
1 a	23	48 848	1 a	24	57 918
b	–	31 168	b	–	62 492
2 a	21	13 342	2 a	24	45 329
b	–	12 131	b	–	13 035
3 a	27	14 120	3 a	26	28 666
b		26 888	b	–	27 404
4 a	24	18 235	4 a	25	35 802
b	–	46 065	b	–	15 684
5 a	26	35 841	5 a	26	8 589
b	–	36 773	b	–	25 349
6 a	24	19 001			
b		17 290			
7 a	38	33 469			
b	–	37 558			

squares divided by 2N (N = the number of pairs in the series) The mean intrapair variance for monozygous twin was 91.3×10^6 and for dizygous twins 175.1×10^6 cpm² An F-test showed that there was no significant difference between these mean variances

The conclusion to be drawn on the basis of this part of the study is that environmental factors probably overrule genetic influence

An Autoradiographic Experiment

The large variation in PHA induced DNA synthesis in lymphocytes could either be due to variations in the degree of synthesis in the individual cells or to variations in the number of cells stimulated by PHA In order to study this problem, the following experiment was carried out Eight normal persons were studied They had been previously tested and were known to cover a wide range of DNA synthesis values Triplicate cultures were established as indicated above After incubation for 46 hours and addition of tritiated thymidine throughout the last three hours aliquots were removed to be used for liquid scintillation counting and autoradiography From each culture 100 cells were counted and the mean percentage of grain covered cells in each person were calculated These percentages were compared with the mean number

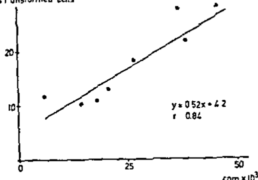


Fig 4 ³H thymidine uptake in PHA stimulated lymphocytes from 8 persons measured by liquid scintillation (cpm × 10³) and autoradiography (per cent transformed cells) The regression line are shown

of cpm obtained by liquid scintillation (Fig 4) The figure shows a good correlation between the number of transformed cells and the tritiated thymidine uptake by the cultures, thus indicating that the variations in PHA stimulation measured by thymidine uptake, is related to the number of cells participating in the DNA synthesis

DISCUSSION

Variations in the lymphocyte response to PHA has been described by several workers The degree of transformation has been found to be influenced by many pathological conditions such as malignant disease (*Silk* (22), *Sample et al* (19) *Hagen & Froland* (6)), multiple sclerosis (*Saunders et al* (20)), Hodgkins disease (*Hatemann* (8)), and certain viral diseases (*Johnson et al* (10)), *Ol*[illegible] *et al* (14) Response has also been found to be depressed in patients with previous myocardial infarction (*Hagen & Froland* [illegible]) and in cases of normal pregnancy (*Pur*[illegible] *et al* (17)) Many studies (*Sample et al* (19), *Richter & Naspitz* (18), *McIntyre & Cole* (11)) has also shown a large variation between apparently normal persons One of the most important factors seems to be age, as previously reported by *Pisciotta et al* (16), *Heine et al* (9), *Sutherland et al* (23)

In our study too the relation between transformation, as expressed by tritiated thymidine uptake, and age seems to be approximately linear But apart from this source of variation, other factors must be in evidence and influence the degree of transformation as can be seen in Fig 2 One of these factors could be sex but we have shown that there was no significant difference between women and men

It could be argued that a large part of the variation could be ascribed to day to-day variations, either in the experimental conditions or in the individuals (*Schellkens & Eijsvoogel* (21)) Our experiments have shown that there is a daily variation, a variation which even is larger than the variation between parallel culture from one blood sample But it also appears that the *level* of transformation in an individual does not change during the time studied A person in whom the level of transformation is high does not have periods with low values Thus, it must be concluded that the level of transformation is individually determined at a given age

It might be expected that the levels were genetically determined *Williams & Benacerraf* (25) found that the ability of mouse spleen cells to respond to concanavalin A or PHA was determined by genetic factors These experiments were carried out in inbred mice living under laboratory conditions Our study of twins failed to show a genetic influence on the response of lymphocytes to PHA, hence, it may be reasonable to suggest that the level of transformation in man mainly is determined by exogenous factors These factors must have been in evidence in the subjects concerned at some time earlier in their life than the time of our investigation and may have a rather constant influence, as only relatively small fluctuations were noted over periods covering from 46 to 390 days

Comparison of the results obtained by the two methods used for the determination of DNA synthesis, i e autoradiography and liquid scintillation counting has given evidence that the level of thymidine uptake is proportional to the number of cells partic-

ipating in the transformation This observation should be seen in the light of the fact that two populations of lymphocytes exist, B (bone marrow) and T (thymus) cells (*Parrot & de Sousa* (15)), and that only T-lymphycytes seem to be able to respond to PHA by DNA synthesis

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ANALYSIS OF C1 SUBCOMPONENTS IN CELL CULTURE MEDIA OF HeLa CELLS AND DIPLOID HUMAN FETAL LUNG FIBROBLASTS

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HeLa cells and diploid human fetal lung fibroblasts in culture synthesize an esterase which is immunochemically and enzymatically identical with C1s of human serum No C1q was found in the purified preparations or the concentrated crude cell culture fluids investigated In the presence of Ca⁺⁺ the C1s of the cell cultures complexed with a substance, also present in the culture medium, possibly being C1r

Three esterase activities were separated from serumfree fluid of non infected HeLa cell cultures (*von Zeipel* 1970) One of these esterases resembled activated C1s Such esterase activities were demonstrated also in cultures of various other animal cells and found to be synthesized by the cells even during prolonged cultivation in serum free media (*von Zeipel* 1972) In the present paper two preparations one from the culture medium of HeLa cells and the other from diploid human fetal lung fibroblasts were analyzed further and compared with C1s and C1q of human serum

MATERIAL AND METHODS

Cell cultures Serum free media from cell cultures were treated as described earlier (*von Zeipel* 1970 1972) Preparation C was a pressure-dialysed concentrate (100 fold) of medium of HeLa cell cultures Preparation D was a similar concentrate (250 fold) of medium of human diploid fetal fibroblast cultures Preparation A was a fraction, containing most of the ATEe activity obtained on chromatography of preparation C on hydroxylapatite Preparation B was the corresponding fraction obtained on chromatography of preparation D

Purified preparations were made of C1s according to *Haines & Lepow* (1964) *C1q* according to *Yonemasu & Stroud* (1971), *C4* partially purified was prepared from euglobulin as described by *Sjoholm & Laurell* (in press), *C1 inactivator* (C1 IA) according to *Pensky et al* (1961)

Antisera to human C1s C1q C1 IA and C4 were prepared by immunizing rabbits with the purified preparations and absorption of the antisera to apparent monospecificity in conventional immunoassays

ATEe (N-acetyl L-tyrosine ethyl ester) hydrolyzing capacity was estimated by pH stat titration, as described by *Laurell & Sjöholm* (1966)

Inhibition of the ATEe hydrolyzing capacity by C1 IA was determined according to *Laurell et al* (1965)

Crossed immuno-electrophoresis was performed according to *Laurell* (1965)

C1q was estimated with the latex agglutination test (*Ewald & Schubart* 1966)

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RESULTS

The purified HeLa cell and the fibroblast preparations, preparation A and preparation B hydrolysed ATEe corresponding to 2 and 80 CIs units per mg of protein, respectively; the high content of protein of prepartion A reflects the fortification of the HeLa cell medium with Bacto tryptose, the high molecular components of which were difficult to eliminate by the purification procedures Addition of CI IA to the reaction mixture in a final concentration of 1 1 U/ml inhibited the esterolytic capacity of preparation A and preparation B to the same degree as it did with CIs in that 1 U of CI IA inhibited about 10 U of preparation A and of preparation B (Table 1)

TABLE 1 *Inhibition by CI IA of ATEe Hydrolyzing Activity of Preparation A and Preparation B*

	Preparation A (HeLa)	Preparation B (lung fibroblasts)
ATEe hydrolyzing capacity	40	22
ATEe hydrolyzing capacity after addition of 1 1 U CI IA	27	10
Expected activity after addition of 1 1 U CI IA	29	11

Preparations A and B and purified CIs were investigated by double diffusing gel precipitation against specific anti CIs, and anti C1q, respectively With anti CIs the preparations gave single precipitation lines identifying with each other No C1q was detected in any of the preparations using a specific anti-C1q (Fig 1)

The failure to detect C1q in the culture fluid preparations prompted us to try a more sensitive technique the latex agglutination test Purified C1q at about 1 μg/ml agglutinated the latex particles No agglutination was obtained with undiluted preparation A or preparation B

Concentrated crude culture fluids (preparation C and D) contained CIs No C1q was detected immunochemically or by the latex agglutination test Nor was C1q detectable by hemolytic assays In addition no C1q was found in extracts of sonicated cells By hemolytic assays also C1r besides CIs, was shown in the crude and the purified cell culture media (*G B Naff & G von Zeipel, unpublished observation*)

CIs and preparation B were analyzed by

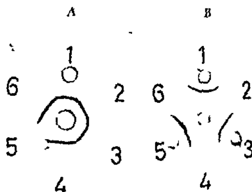


Fig 1 Gel diffusion test of preparation A and B
A Anti CIs in the central hole *1* preparation A, *2, 4, 6* CIs, *3* preparation B *5* buffer
B Anti C1q in the central hole *1, 3, 5* C1q preparation, *2, 4* preparation A, *6* preparation B

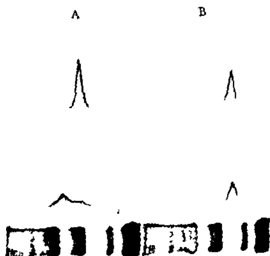


Fig 2 Crossed immunoelectrophoresis with anti CIs in the agarose gel
A Separation in the presence of Ca Above CIs from human serum *Below* preparation B
B Separation in the presence of EDTA *Above* CIs from human serum *Below* preparation B
For reference the electrophoretic pattern of normal serum is shown

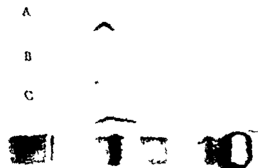


Fig 3 Crossed immunoelectrophoresis with anti C4 in the agarose gel
A C4 incubated at 37°C for 30 minutes.
B C4 incubated with CIs (final concentration 7 U/ml) at 37°C for 30 minutes
C C4 incubated with preparation B (final concentration 7 U/ml) at 37°C for 30 minutes
For reference the electrophoretic pattern of normal serum is shown

crossed immunoelectrophoresis with anti CIs in the agarose gel The electrophoretic separations were performed in the presence of Ca^{+} or of EDTA 2 10 $^{-3}$M CIs gave a quite dominating symmetrical peak under both conditions In the presence of Ca^{++} the protein migrated slower than in the presence of EDTA Analysis of preparation B in the presence of EDTA resulted in a single precipitation peak in the same position as CIs investigated under the same conditions With Ca present during the electrophoretic separation of preparation B two precipitations peaks were found showing a reaction of identity One peak had the same position as CIs analyzed in the presence of Ca while the other peak had a slower electrophoretic mobility (Fig 2)

Partially purified C4 was incubated with CIs and preparation A and B respectively C4 was then analyzed by crossed immunoelectrophoresis using a specific anti C4 All three preparations gave rise to the same electrophoretic conversion pattern of C4 (Fig 3

DISCUSSION

Various mammalian cells in culture produce an esterase that hydrolyses ATEe inactivates the hemolytic capacity of the fourth component of complement and forms hemolytically active C1, when combined with C1q and C1r This enzyme was synthesised even by cells which had been cultivated for years in serum-free medium (*von Zeipel* 1970, 1972)

In the present investigation it was shown that the ATEe hydrolyzing capacity of preparations from culture media of HeLa cells and human fetal fibroblasts was inhibited by CI IA of human serum to the same degrees as was found for serum CIs Gel precipitation analysis showed that both types of cells produced a protein with antigenic properties indistinguishable from those of CIs prepared from human serum Similar amounts of preparations A and B and of CIs as estimated by the ATEe hydrolysing capacity gave the same pattern of conversion of C4

Thus all evidence so far obtained indicates that CIs is formed by the two cell types during cultivation

Hemolytic assays showed that preparation B besides CIs also contained C1r but no C1q (*G B Naff* & *G von Zeipel*, unpublished observation) The retardation of the electrophoretic mobility of CIs in the presence of Ca^{+} compared to the migration rate in the presence of EDTA indicates a binding of Ca^{++} to the CIs molecule Ca^{++} influenced the CIs of preparation B in that two CIs peaks appeared on crossed immunoelectrophoresis, indicating a formation of a complex between CIs and a component present in the culture media The possibility may be considered that the slowly migrating peak (Fig 2) consists of a complex between CIs and C1r with Ca^{++} as a ligand This interpretation is in accordance with the findings with hemolytical assay techniques that the preparations contained besides CIs also C1r

Attempts to demonstrate C1q in the purified preparations and also in crude concentrated cell culture fluids by both immunochemical assays and by the latex agglutination test were unsuccessful It therefore seems that neither the partially purified preparations nor the concentrated crude cell culture fluids did contain appreciable amounts of C1q On

crossed immunoelectrophoresis with anti C1s in presence of Ca^{++} serum macromolecular C1, comprising a complex of C1q, C1r, C1s, forms a precipitation peak located at the site of application for the electrophoresis (*de Bracco & Stroud* 1971, *Laurell*, to be published), thus showing a distinctly different electrophoretic mobility than the slow peak seen in Fig 2 The cell culture preparation therefore hardly contained complete macromolecular C1

Kosa & Fust (1970) reported anticomplementary substances produced by KB cell cultures They showed a substance that had several properties in common with CI On Sephadex G 200 gel filtration the CI like substance was eluted as a protein with a molecular weight above 200 000 This contrasted with the data given by *von Zeipel* (1970) who reported a molecular weight of 115 000 of the CIs of HeLa cell cultures The discrepancy might be explained by differences in purification procedures The results obtained by *Fust & Kosa* (1972) suggest that the KB cells produced C1s and possibly also other subcomponents of C1

Colten et al (1968) found in tissue culture that hemolytically active C1 was synthesized by cells in the colon and ileum In their experiments the synthesis of macromolecular, hemolytic active CI might be the net result of the possible synthesis of the C1 subcomponents by different cells existing together under the experimental conditions used

Stecher et al (1967) found C1q to be synthesized in various organs and concluded that macrophages were probably the responsible cells *Stroud et al* (1970) reported an imbalance between the level of C1s and C1q in serum from patients with lymphopene hypogammaglobulinemia in that C1s was normal while C1q was markedly reduced They suggested that different types of cells might synthesize the different subcomponents of the macromolecular serum C1 complex

The finding of the present investigation may support the view that different cells produce C1q and C1s

Pondman et al (1968) described a child with systemic lupus erythematosus with a selective deficiency of C1 hemolytic activity owing to deficiencies of C1r and C1s *Pickering et al* (1970) reported chronic glomerulonephritis in a child whose serum lacked C1 hemolytic activity The serum was deficient in C1r and had an abnormally low level of C1s They proposed, as one possible explanation that the aberration of the C1 subcomponents might reflect a direct linkage of synthesis of C1r and C1s

It is known (*Naff et al* 1964, *Laurell & Siboo* 1966, *Sassano et al* 1972) that the separated subcomponents of C1 can complex in the presence of Ca^{++} *in vitro* to form hemolytically active, macromolecular C1 The difference in molecular size andf hemolytic capacity between serum C1 and the complex formed on recombination of the three subunits seems to reflect an assembly problem of macromolecular C1 *in vitro* the reason for which is not known If the subcomponents of C1 are synthesized by different cells, this may suggest a possible basis for a deficient *in vivo* assembly of the subcomponents

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TABLE 2 *The Effect of Neuraminidase (Viral or RDE) on the Agglutinability of Trypsinized Human Erythrocytes by Influenza A2 and Adeno Types 5 and 9 Viruses*

Cell System	HA titres of following viruses			
	Influenza		Adeno	
	A2/Hong Kong/68	X-7	type 9	type 5
Rat*	NT	NT	256	256
Fowl*	512	512	NT	4
Human	256	256	256	32
Human & trypsin	256	256	256	512
Human & RDE	< 4	< 4	< 2	256
Human & viral neuraminidase	< 4	< 4	< 2	256
Human & trypsin & RDE	< 4	< 4	< 2	512
Human & trypsin & viral neuraminidase	< 4	< 4	< 2	30 000

* Rat and fowl cells used to monitor background titres of adeno and influenza viruses respectively
NT — Not tested

NANA Some lysis of the erythrocytes occured at the lower dilutions probably due to the acidic nature of these dilutions Because of the exclusively inhibitory nature of the NANA, it might be reasonable to implicate this particular sugar in the red cell receptor structure Tryptic digestion of human erythrocytes releases a glycopeptide containing NANA terminally linked to a penultimate N acetyl glucosamine residue (15) Thus it appears that these surface sialo glycopeptides are not the exclusive receptors for the adenovirus

The nature of the erythrocyte receptor was studied further by treating the cell with viral or bacterial neuraminidase Asian influenza virus neuraminidase from the recombinant X 7 was prepared as previously described (1) and further purified by molecular sieve chromatography on 4 per cent agarose gel Bacterial neuraminidase from *Vibrio cholerae* (RDE) was purchased from N V Philips Roxane, the Netherlands and from Burroughs Wellcome Beckenham England The results obtained with the two RDE products were the same Various dilutions of viral or bacterial neuraminidase were mixed with equal volumes of 1 per cent erythrocyte suspensions and the mixtures held for 1 hour at room temperature The dilution that rendered the cells inagglutinable by the biological control viruses A2/Hong Kong/68, the X 7 recombinant and the adenovirus type 9 serotype which completely agglutinates human O red blood cells and whose receptors are known to be sensitive to RDE treatment (14) was used for further studies It was not possible to test rat erythrocytes in this manner because they were rendered autoagglutinable by this treatment Against the background of appropriate controls it can be seen that RDE treatment of trypsinized cells which cleaves off NANA failed to alter the agglutinability of the cells by type 5 adenovirions (Table 2) On the other hand viral neuraminidase which cleaves off N, O, diacetylated neuraminic acid (12) as well as NANA resulted in a marked increase in titre of the adenovirus These different neuraminidases also have different specificities as regards their ability to split the various types of linkages of NANA to adjacent carbohydrate (4 7, 8) Since human material has never been shown to carry sialic acids other than NANA, this differential behaviour is probably a linkage phenomenon These observations would appear to be in harmony with those of *Boulanger et al* (3) who showed that treatment of host (KB) cells with *Diplococcus pneumoniae* neuraminidase resulted in a significant increase in adsorption of type 2 virions (group III) to those cells

The data indicate that NANA is implicated in the agglutination of human group O erythrocytes by group III adenovirions As the most likely receptor NANA would appear to occur on the cell as exposed glycopeptide (15) or partially occluded glycolipid (11) located in cellular crenellations and invaginations

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Thanks are due to Prof *G Belyavin* and Dr *D S Pepper* for helpful discussions The technical assistance of Miss *Ylva Gollmar* is gratefully acknowledged

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STUDIES ON THE NITROBLUE-TETRAZOLIUM STAINING INDUCED IN HUMAN NEUTROPHILS BY BACTERIAL PRODUCTS

Christian Koch

In patients with acute bacterial infections, an increased percentage of the peripheral neutrophil granulocytes reduce the histochemical dye, nitroblue tetrazolium (N B T), to blue black formazan *in vitro* (2, 5 7) Several experimental models demonstrate a possible link between bacterial infections and increased N B T staining, thus this can be induced *in vitro* by phagocytosis of bacteria, by stimulation with endotoxin (8), and with bacterial culture filtrates (6) This mechanism has, however, yet to be accurately outlined and among the many unanswered questions is the possible role of extra cellular factors in this interaction Work in this laboratory had revealed that culture filtrates from certain strains of bacteria cause increased N B T staining in neutrophils from all individuals tested if added to heparinized blood whereas filtrates from other strains of which *Diplococcus pneumoniae* is one, will cause increased N B T staining in some individuals but not in all Similar individual specificities have been recorded previously (6) and found to be consistent over a prolonged period On the assumption that host specific cellular or extracellular factors might be of significance for the increased N B T staining caused by some bacterial filtrates the following studies were done

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Materials and Methods

A patient was selected for further studies because screening of his leucocytes with different filtrates revealed a moderate response to *Diplococcus pneumoniae* (Pneumococcus) filtrate to which the leucocytes of several normal control persons showed no, or only a slight response The patient was a seventy year old male suffering from chronic bronchitis, who had experienced several episodes of lower respiratory tract infections due to Pneumococci Heparinized blood was obtained from the patient and a normal control who failed to respond to stimulation with Pneumococcus filtrate upon repeated testing Plasma and serum were also isolated from freshly drawn heparinized or unstabilized blood from the patient and the control Culture filtrates from freshly isolated strains of bacteria from infected patients, and one laboratory strain (*Staph aur* 502 A) were obtained by overnight growth in meat infusion broth Pneumococci were grown in this medium enriched with 10 per cent horse serum The cultures were centrifuged at 5 000 rev/min for 30 minutes, passed through 0 45 μ Millipore filters, and stored at −20°C

For direct tests 5 μl filtrate was added to 0 1 ml heparinized blood For pre incubated tests 10 μl filtrate was added to 0 5 ml plasma or serum and incubated at 35°C for 60 minutes The plasma or serum was then added to blood cells from either the patient or control in the following manner Portions of 0 5 ml heparinized blood were washed three times at low speed centrifugation in gelatinized, heparinized Hank s balanced salt solution adjusted to physiological pH The final cellular pellet was then resuspended in the 0 5 ml portions of pre-

TABLE 1 *Effect of Various Bacterial Culture Filtrates on the N B T Staining of the Patients Neutrophil Granulocytes*

Bacterial Culture	Percentage of N B T stained neutrophils	
	Pre in-cubated	Direct
Diplococcus pneumoniae	40	13
Staphylococcus aureus	11	9
„ „ 502 A	10	5
Eschericia coli	6	4
Pseudomonas aeruginosa	4	2
Neisseria meningitidis	11	7
Klebsiella pneumoniae	26	17
Control — filtrate		2

Pre incubated 10 μl filtrate per 0 5 ml plasma incubated at 35°C for 60 minutes before addition to washed homologous cells
Direct 5 μl filtrate per 0 1 ml heparinized blood

incubated plasma or serum by gentle mixing In some experiments plasma was inactivated prior to pre incubation with the filtrate by heating at 56°C for 30 minutes In other experiments plasma and serum was centrifuged after pre incubation in a Sorvall RC2 B centrifuge at 20 000 rev/min corresponding to 48 200 x G for 30 minutes at 4°C, before addition of the supernatant to the blood cells in order to remove any precipitable immune complexes formed

The N B T tests were done according to the method of Park et al (7) with three modifications 1) blood N B T mixtures were incubated at 35°C for 30 minutes 2) incubations were carried out in acryl haemagglutination trays, and 3) cover slip smears were counterstained with May Grunwald Giemsa stain Two smears were made for each test and 250 neutrophils from the two smears were counted and evaluated

Results and Discussion

In direct tests (Table 1) the patient responded to several bacterial filtrates with an increased percent

TABLE 2 *Specificity of Plasma-Source and Effect of Heat Inactivation on the N B T Staining Induced by Pre Incubation with Pneumococcus Filtrate*

Source of leucocytes	Source of plasma	Heat inactivation	Pre incubation	Percentage of N B T stained neutrophils
Patient	patient	–	–	4 4
Control	control	–	–	6 8
Patient	patient	–	+	47 2
–	control	–	+	3 6
Control	patient	–	+	53 2
–	control	–	+	3 6
Patient	patient	+	+	2 0
–	–	+	+ in presence of 20 per cent control plasma	19 6

TABLE 3 *Effect of High-Speed Centrifugation on the N B T Staining Induced by Pre Incubation of Patient Plasma and Serum with Pneumococcus Filtrate*

Source of leucocytes	Pre-incubation	Centrifugation at 48 200 x G	Percentage of N B T stained neutrophils	
			Plasma	Serum
Patient	–	–	0 4	1 2
	–	+	0 0	1 6
–	+		64 4	43 6
–	+	+	3 2	16 0
Control	–	–	8 0	8 8
–	–	+	7 6	7 6
–	+	–	78 8	58 8
–	+	+	15 2	20 8

age of N B T stained cells, notably to Pneumococcus and *Klebsiella pneumoniae* This response was enhanced when plasma was pre incubated with filtrate, thus enhancement cannot be due to an increased concentration of filtrate In another experiment (Table 2) patient and control plasma were pre-incubated with Pneumococcus filtrate and added to washed homologous and heterologous cells Pre incubated patient plasma caused increased N B T staining in both patient and control neutrophils, whereas pre incubated control plasma failed to do so in either cell population In the same experiment it was found that heat inactivation of the patient's plasma prior to pre incubation completely abolished the increased N B T staining induced in homologous neutrophils but this could be partially restored by carrying out the pre-incubation in the presence of 20 per cent control plasma In a third experiment (Table 3) it was found that high speed centrifugation of pre incubated patient plasma, carried out to remove any precipitable immune complexes possibly formed, caused appreciable reduction in the power to induce increased N B T staining in either cell population although some power of induction remained in the supernatant The same pattern was found if serum from the patient was used instead of plasma (Table 3) However while more power of induction was present in pre incubated plasma than in serum from the patient, then more power of induction remained in the supernatant from serum than in that from plasma following high speed centrifugation

A possible interpretation of the present findings is that bacterial antigens reacted with antibacterial antibodies present in the patient to form soluble and precipitable immune complexes that subsequently reacted with the neutrophils in a manner that induced increased N B T staining Non specific heat labile factors seemed to be critical, either for the formation of complexes or their reaction with the neutrophils (Table 2) There is no clear explanation for the difference of behaviour between plasma and serum in this respect (Table 3) but it might be that fresh heparinized plasma in this situation favoured the formation of precipitable over soluble immune complexes compared to fresh serum and that precipitable complexes are more effective than soluble ones in inducing increased N B T staining The correlation of size of immune complexes formed *in vitro* to several biological activities such as rate of uptake by neutrophils, release of lysosomal enzymes from neutrophils and ability to fix complement has been reported (3), on the other hand release of lysosomal enzymes from neutrophils upon exposure to immune complexes *in vitro* has been noted even in the absence of actual uptake (4) The present findings indicate that uptake of immune complexes, or contact with complexes formed *in vitro* by reaction of bacterial antigens with specific antibodies can non specifically induce increased N B T staining of neutrophil granulocytes, and that this mechanism is dependent upon the presence of non specific heat labile factors It remains, however, to be established whether such a mechanism may operate *in vivo* to account for the increased N B T staining of neutrophils from patients with bacterial infections

False negative N B T tests have been noted by others (1) in some cases of acute bacterial meningitis, and also in this laboratory, and absence of bacteraemia has been proposed to account for this finding (1) Another explanation is offered by the present findings, namely that absence of bacterial antigens in the blood stream, or absence or low titres of antibodies capable of reacting with these antigens, and subsequently with neutrophils might in some instances account for unexpected false negative N B T tests This does not exclude that some strains of bacteria may produce products that are capable of direct stimulation of neutrophils causing increased N B T staining Further studies of the mechanism of *in vitro* induction of increased N B T staining of neutrophils by bacterial products seem relevant for the clinical interpretation of the N B T test now so widely used as an important adjunct in the early diagnosis of acute febrile disorders

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CULTIVATION OF PATHOGENIC *TREPONEMA PALLIDUM* IN CHAMBERS SURGICALLY IMPLANTED IN EXPERIMENTAL ANIMALS

T Rathlev

In spite of considerable experimentation during the last few decades treponematologists have not yet succeeded in cultivating the pathogenic strains of *Treponema pallidum in vitro* This has been a serious handicap in obtaining adequate amounts of pure culture of the organisms necessary for studying their antigenic components, the nature of immunity produced in the host animal and the production of a purified effective vaccine against treponemal diseases like syphilis or yaws

The growth of *Treponema pallidum* has been studied in several species of animals and the rabbit seems to be the animal of choice The organisms grow and multiply well in the testicles of rabbits and are usually extracted 8 12 days after inoculation Spermatozoa and blood cells which are much heavier than the treponemes are removed by low speed centrifugation This treatment yields treponemal suspensions which are used for infecting animals and as an antigen in the serological tests such as TPI, FTA and passive hemagglutination However these suspensions of treponemes even when killed or attenuated are not quite suitable for vaccination in humans due to the remnants of host tissue—particularly testicular tissue which might produce tissue damaging immune response, as several injections are necessary to attain adequate immunity

Removal of host tissue and host serum proteins can be achieved to a great extent by density gradient centrifugation in potassium tartrate sucrose and cesium chloride or by continuous particle electrophoresis However, unlike some viruses which have been successfully purified by some of these procedures *T pallidum* is rendered non virulent, possibly due to osmotic shock or toxicity of the environmental material

Since the virulence and motility of *T pallidum* can be preserved for years in liquid nitrogen, the problem in treponematoses research is really not the maintenance of virulence of the organisms outside the host animal, but to obtain fairly large numbers of treponemes freed from host tissue and proteins especially the antitreponemal immunoglobulins *In vitro* cultivation of these organisms is undoubtedly the best solution to the problem This of course will necessitate a great deal of experimentation to study the requirements of different nutrients, and optimal conditions of pH, temperature and mixture of gases While such studies are in progress the present investigation was undertaken to cultivate *T pallidum* in animals under new experimental conditions The idea of surgical implantation of chambers in animals to grow *T pallidum* emanated from a report for culturing a virus in chambers inserted in experimental animals (9) A modification of this procedure was tried successfully for the cultivation of *Neisseria gonorrhoeae* in animals (1)

MATERIAL AND METHODS

The experimental animals used were young adult rabbits of New Zealand strain, and inbred guinea pigs with body weight ranging from 500 to 700 g All animals were bled before implantation of chambers by cardiac puncture, and the serum was tested by serological methods to establish non reactivity to treponemal antigen

The surgical implantation of chambers was carried out in rabbits and guinea pigs of both sexes As previously reported (1), a sterile polyethylene practice golf ball was inserted in the subcutaneous, dorso-lumbar region of the anesthetized rabbit Smaller polyethylene, round chambers (inner diameter 1 5 cm, wall thickness 1 mm) were used for similar implantation in the guinea pigs. These smaller chambers had holes in their walls just as the bigger ones and could be sterilized Guinea pigs

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were anesthetized with ether and incisions were made on each side of the shaved, dorso lumbar area The skin over the subcutaneous region was stretched with the forceps to produce a pouch about 3 cm in length from the incision, and the sterile polyethylene chamber was inserted in the pouch on each side The incisions were closed by suturing and use of wound clips The clips were removed a week later The incisions healed completely after 4 weeks in the rabbits and in 3 weeks in the guinea pigs At this stage the chambers were covered with a coating of fibrous connective tissue accompanied by the production of a yellowish transudate These observations were made by making a cross section through the chambers implanted in some of the animals

Treponemal inoculum injected in the chambers implanted in the rabbits, was cultivated in rabbit testicles, while the inoculum used for infecting the chambers inside the guinea pigs was cultivated either in rabbit testicles or guinea pig testicles

After removal of ca 1 ml transudate from the bulging area covering the chamber, 1 ml treponemal inoculum (ca 10 14 $\times 10^7$ organisms) per chamber in the rabbits and 0 5 ml inoculum (ca 5 7 $\times 10^7$ organisms) per chamber in the guinea pigs was introduced with a hypodermic needle and syringe From the 6th day after infection, few microlitre samples were removed from the chamber for examination and a count of the treponemes by darkfield microscopy On the same day, 5 ml blood samples from the rabbits and 1 or 2 ml from the guinea pigs were removed by cardiac puncture to obtain sera for determining the antibody response by different serological methods This procedure was repeated every second day for a total period of 2 weeks in the rabbits and 7 or 8 weeks in the guinea pigs A group of 10 guinea pigs was infected, bled and punctured for treponemal samples on the same day since 2 or 3 animals from the group died during the 8 week period as a result of repeated cardiac punctures Therefore, a mean value of all the results obtained from the 10 animals was considered as a representative response to the treatment given

TPI, FTA ABS, and passive hemagglutination test with the treponemal antigen were carried out by the routine procedures used in our laboratory (4 6 7) Presence of the anticardiolipin antibody was determined by the complement fixation test using cardiolipin antigen

RESULTS AND DISCUSSION

This investigation was carried out using 50 guinea pigs and only four rabbits The guinea pig was preferred as a better animal for this study on the basis of results of an investigation from our laboratory (8), the results of which showed that *T pallidum* multiplied and maintained its virulence in guinea pigs for 6 8 weeks without producing discernible syphilitic lesions

In the study reported here, the number of treponemes increased about 10 fold by the 6th week after infection and decreased rather abruptly with a concomitant fall in the immobilisin However a booster inoculum (ca 0 2 ml) injected in the chamber at this stage increased the growth of the treponemes accompanied by an increase in the immobilisin The treponemal suspension from the chambers was removed once every week to infect normal rabbits intratesticularly Production of the usual syphilitic orchitis indicated that the treponemes maintained their virulence for the 8 week experimental period, and there was no species barrier in their infectivity

In earlier studies (2, 5 10), guinea pigs had been used as experimental animals to produce syphilis However, since the infection was almost asymptomatic, and no investigation of the antibody response or the multiplication of the treponemes was carried out, the authors concluded that the guinea pig offers no advantage as an experimental animal over the rabbit This claim is justified so far as the yield of treponemal suspension in one extraction is concerned However the duration for obtaining viable and virulent treponemes from a guinea pig is almost indefinite This animal seems to harbour treponemes *in vivo* in a state of 'symbiosis' without production of syphilitic lesions and the resulting anticardiolipin antibody It has been previously reported (3) that rodents produce the immobilisin, but no anticardiolipin antibody as a result of infection with *T pallidum* The guinea pig showed similar response The immobilisin reached its peak value (100 per cent positive) from 10 to 12 days after infection, and persisted at this level for at least 4 weeks On the other hand FTA ABS and passive hemagglutination tests were negative for nearly 5 to 6 weeks after infection and became only weakly positive Cardiolipin complement fixation test for the anticardiolipin antibody was negative throughout the experimental period

This unusual response which is not observed in the rabbits provided means of isolating from the guinea pig serum immobilisin which might be very valuable in studying the mechanism of its bactericidal action in combination with the complement

Cultivation of *T pallidum* in chambers implanted in guinea pigs also presents the possibility of studying the life cycle of the organism for a fairly long period, since frequent removal of treponemal suspensions is not harmful to the animal This is not possible in the rabbit which is very susceptible to syphilitic lesions which are accompanied by the production of antibodies in the blood and hyaluronic acid at the site of the lesions These products may affect the viability of the treponemes which therefore have to be extracted 8 12 days after the infection

CONCLUSIONS

The surgical implantation of chambers presents a new technique of cultivation of *T pallidum* in animals It has some advantages since removal of test samples of the organisms is easier and can be done frequently without injury to the animal Poly ethylene being biologically inert, does not evoke an immunological rejection of the chambers which therefore can be retained in the animal almost indefinitely

The guinea pig seems to be a very good experimental animal for studying the growth cycle of *T pallidum* and some of the antibody responses induced by it for a much longer period of time than is possible in rabbits

This new technique may provide possibilities to cultivate other pathogenic strains of treponemes such as *T pertenue* and *T carateum* either in smaller animals or in primates to study the course of diseases like yaws or pinta in experimental animals.

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MEASUREMENT OF C1 INACTIVATOR (ALPHA 2 NEURAMINOGLYCOPROTEIN) ON HUMAN BLAST CELLS IN BLAST LEUKAEMIA

Kurt Osther and Ryan Linnemann

Osther & Linnemann (4) have recently reported a coating of C1 inactivator of cultured human carcinoma cells C1 inactivator has now been assayed in *in vitro* experiments on blood marrow cells from patients suffering from blast leukaemia

Conjugated anti C1 IA FITC (anti C1 inactivator—fluorescein isothiocyanat) was used for these experiments as reported in our paper (4)

The material consisted of sternal bone marrow from a total of 14 patients who had been admitted to the Medical Department the Finsen Institute or the Medical Department, Kommunehospitalet Copenhagen Among these 14 patients 10 were suffering from acute myeloid leukaemia 3 from acute lymphoid leukaemia and 1 from acute blast leukaemia The control material consisted of sternal bone marrow from 6 patients with lymphadenitis veneficium medicamentalis polycythaemia, neurological disorder, sideropenic anaemia and thrombocytopenia, respectively

About 1 ml of blood marrow was placed in sterile tubes coated with 100 units of heparin per ml and cooled for 3–4 hours in a refrigerator at + 4° C The blood was then centrifugated in a Christ II KS refrigerated centrifuge at 800 rev/min for 15 minutes at a temperature of + 4° C

To obtain a higher amount of white blood cells, 50 microlitres were taken from the intermediate layer All bone marrow specimens have been subjected to routine examination in the pathological departments. They were incubated directly with 0 5 ml anti C1 IT FITC for 30 minutes at 37° C in an incubator

The excess of anti C1 IA FITC was eluated and washed with 0 15 M saline at room temperature

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by serial centrifugation, 800 rev/min, 20° C, 4 times

After the last centrifugation the cells were re suspended in 0 1 ml 0 15 M saline One drop of the suspension was mixed with one drop of glycerin direct on a slide which subsequently was sealed Shortly after sealing the cells were measured by a Leitz cytophotometer MPV I as reported (4)

Myeloid cells and haemocytoblasts exclusively were selected by phase contrast microscopy and measured The resulting photo current from the immunofluorescence measurements of anti CI IA-FITC on the scored cells are expressed in abitrary working units (Table 1)

TABLE 1 *Anti CI Inactivator FITC Immuno fluorescence Measurements on Human Cell Populations Using a Leit MPV I Cytophotometer*

Human cell populations	Cytophotometric measurements		
	< 30 w u	Between 30 and 40 w u	> 40 w u
Benign	99 %	1 %	0 %
Malignant	37 %	20 %	43 %

The photo current induced by the transmitted fluorescence is expressed in arbitrary working units (1 w u $\sim 2 \times 10^{9}$ Amp) The method is described in detail elsewhere (4)

The human cell populations comprised 14 obtained from patients suffering from blast leukaemia and 6 obtained from healthy donors

Fluorescence scores < 40 w u are considered non specific

Cells coated with CI IA were found in every bone marrow specimen from leukaemic patients On an average, 43 per cent of the cells measured were coated 100 per cent of the cells measured in patients with non neoplastic diseases were not coated with CI IA using 40 w u as border line (4)

In the natural host of murine leukaemia virus *Huebner* & *Todaro* (3) and *Rowe et al* (7) have not been able to find depletion of complement (CF response) in a variety of systems On the other hand, potent complement fixing (CF) antibody against murine leukaemia can readily be induced in heterologous hosts, suggesting that the humoral immune response of the host plays a significant role in the regression phenomenon (1, 2, 5 8) Furthermore *Rich et al* (6) found a relationship between serum complement depletion and regression of murine leukaemia

It is known that serum complement together with antibodies (IgG, IgM) comprise the cytotoxic principle in the humoral immune defence The authors' findings in the different murine leukaemia systems intimate a blocking of the complement system

We have assumed that CI IA is the blocking factor coating the neoplastic cells and, in this way, inhibiting the complement system in its cytotoxic activity

The results concerning carcinomas (4) and leukaemias suggest a coating of malignant cells with CI IA and may explain a protection against the humoral immune system

In 2 follow up assays we found that the CI IA activity on the cells disappeared during regression of the leukaemia induced by cytostatics It is likely to suppose a connection between the disappearance of CI IA and the regression

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ANALYSIS OF THE ELECTROPHORETIC PATTERN OF MYCOPLASMA PROTEINS FOR THE IDENTIFICATION OF CANINE MYCOPLASMA STRAINS

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The influence of medium composition and cultivation time on the electrophoretic pattern of mycoplasma proteins has been investigated It was found that media containing horse serum are unsuitable because increasing amounts of medium protein are harvested together with the mycoplasmas during incubation The medium protein gives rise to unspecific electrophoretic bands which grow more numerous and intense during incubation These disadvantages were not found in a medium containing PPLO serum fraction An examination of the electrophoretic patterns of 25 *M canis* 16 *M eduardii* and 12 *M spumans* strains demonstrated that interspecies differences were more pronounced than intraspecies variations It is concluded therefore that the electrophoresis method may be used as an aid in the classification of mycoplasmas isolated from dogs

The electrophoretic separation of mycoplasma proteins in polyacrylamide gels gives rise to characteristic patterns (5, 8) that have been utilized during recent years as a taxonomic tool in mycoplasmology

However, the method has certain limitations For example, comparison of the patterns may be complicated by the fact that about 20 30 close bands develop by electrophoresis of the cell proteins Furthermore, variations in the patterns of different strains of one species are often seen (4)

The question is to what extent differences in patterns between two species differ from the variations that may exist between different strains of the same species This question can only be answered by examination of the electrophoretic pattern of a greater number of strains from a few different species

The purpose of this work was to elucidate the above mentioned question by examining the electrophoretic patterns of 53 canine mycoplasma strains belonging to three different species In addition, the influence of medium composition and time of cultivation on the electrophoretic pattern was investigated

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MATERIALS AND METHODS

Mycoplasmas The type strains of *M spumans* (PG 13), *M canis* (PG 14), and *M eduardii* (PG 24) were examined together with 50 cloned strains recently isolated from the respiratory and genital tract of 41 dogs Seven of these dogs came from private homes while the remaining came from three kennels, namely 13 14 and 7 from each The strains were identified by means of indirect immunofluorescence (7) and the growth inhibition method (2)

Cultivation media 1) Medium 1 i e Heart infusion broth (Difco), 2 5 per cent (w/v), 90 ml Fresh yeast extract 25 per cent (w/v) 10 ml, Horse serum A (A indicates a particular horse), 20 ml DNA (Sigma), 0 2 per cent (w/v), 1 2 ml

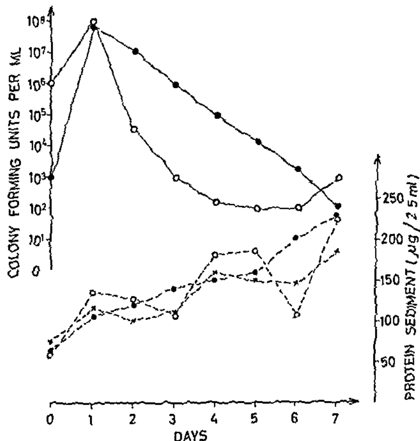


Fig 1 Growth of *M spumans* (PG 13) (●——●) and *M edwardii* (PG 24) (○——○) in medium containing horse serum A Protein sediment from *M spumans* (PG 13) culture (● - - - ●), *M edwardii* (PG 24) culture (○- - - ○), and uninoculated medium (×- - - ×)

Thallium acetate, 10 per cent (w/v), 1 ml, and Na penicillin, 50 000 i u

2) As medium 1, but inactivated at 56° C for 30 min

3) As medium 1, but horse serum A was replaced by horse serum from another horse B

4) As medium 1 but horse serum (20 ml) was replaced by 2 ml PPLO serum fraction (Difco)

The pH level of all media was adjusted to 7 5 The media were centrifuged at 30 000 × g (maximum value) for 60 minutes at 4° C and the supernatants were sterile filtered before use

Preparation of cell extract Mycoplasmas were harvested from liquid cultures by centrifugation in a Sorvall RC-2 refrigerated centrifuge at 27,000 × *g* (*maximum value*) for 30 minutes at 4° C The pellet was washed twice in distilled water After washing the amount of protein was determined according to *L wry et al* (3) Cells were dissolved overnight 4 C in phenol acetic acid water (2 1 0 5 w) using 0 2 ml solvent per mg protein Und ved material was sedimented by centrifugati 15 000 × g (maximum value) for 15 minutes 4 C

Distilled water was used for washing of cells because preliminary experiments comparing distilled water and Sorensen's phosphate buffered saline pH 7 2*, had demonstrated that washing with distilled water gave the highest protein yield The possible influence of the washing method on the electrophoretic pattern of two test strains was examined (*M spumans*, PG 13 and *M edwardii*, PG 24) It applied to both strains that the pattern of cells washed in phosphate buffered saline was found to be similar to the pattern of cells washed in distilled water In the case of *M edwardii* (PG 24) this was the fact even though the number of colony forming units per ml (c f u /ml) was very much reduced following washing with distilled

ried out
ide gels

The composition and polymerization of the gels were as described by *Rottem & Razin* (8) For each gel 100 μl of dissolved protein (0 5 mg) was

* (KH_2PO_4 3 23 g, Na_2HPO_4 $2H_2O$, 10 63 g NaCl, 78 75 g, and distilled water ad 10 000 ml)

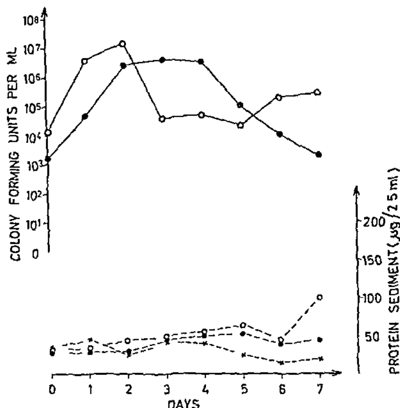


Fig 2 Growth of *M spumans* (PG 13) (●——●) and *M edwardii* (PG 24) (○——○) in medium containing PPLO serum fraction Protein sediment from *M spumans* (PG 13) culture (● ---●), *M edwardii* (PG 24) culture (○- - ○) and uninoculated medium (×- --×)

mixed with 50 µl of a 2.2 M sucrose solution in 5.8 M acetic acid and placed on top of the gel The protein sucrose mixture was overlaid with 0.5 ml of 12.5 M acetic acid Both anode and cathode buffer consisted of 1.7 M acetic acid Electrophoresis was carried out at room temperature for 2 hours with a constant current of 4 mA per gel During this period the proteins migrated towards the cathode end of the gel The gels were stained with 1 per cent (w/v) Amido Black 10B in 7 per cent (v/v) acetic acid for two hours Thereafter they were rinsed under tap water and excessive stain removed electrolytically in 7 per cent (v/v) acetic acid using a direct current of 200 mA

EXPERIMENTS AND RESULTS

Experiment 1 Influence of Media and Time of Cultivation on the Protein Yield and the Electrophoretic Pattern

In order to elucidate this the following experiment was carried out 3 × 400 ml of each of the four media were prepared Two portions were inoculated with *M spumans* (PG 13) & *M edwardii* (PG 24) respectively The cultures were incubated at 37° C together with the third portion which served as uninoculated control The number of c f u /ml and the amount of protein sediment per 2.5 ml centrifuged culture was determined daily during a period of 7 days for each portion Every second day (1, 3, 5, and 7 day), 80 ml of each portion was centrifuged and the sediment prepared for electrophoresis

a Growth and Protein Yield from Media Containing Horse Serum

In Fig 1 are shown the results of determinations of the number of c f u /ml and protein in the experiment with the medium containing horse serum A The results of ex-

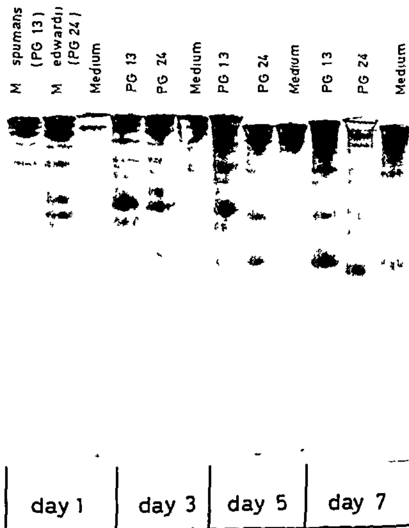


Fig 3 Electrophoretic patterns of protein (0 5 mg per gel) obtained by centrifugation at day 1, 3, 5 and 7 of *M spumans* (PG 13) culture, *M edwardii* (PG 24) culture, and uninoculated medium Culture medium containing horse serum A

periments with the other media containing horse serum were very similar Thus it was characteristic that the number of c f u /ml for *M spumans* (PG 13) and *M edwardii* (PG 24) reached a maximum (10^7-10^8) after one day of incubation After 6 7 days the number of c f u /ml fell to about 10 The protein sediment per 2 5 ml centrifuged culture was found to rise from about 50 μg to about 200 μg during the period of incubation In this respect there was no difference between the inoculated and the uninoculated portions

b Growth and Protein Yield from Medium Containing PPLO Serum Fraction

In Fig 2 are shown the results of determinations of the number of the c f u /ml and protein in the experiment with the medium containing PPLO serum fraction It appears from the figure that the number of c.f u /ml in both cultures reached a maximum (10^6-10^7) after 2 3 days of incubation The number of c f u /ml declined slowly to 10^4-10^5 at day 7 The amount of protein sediment per 2.5 ml centrifuged culture was found to vary

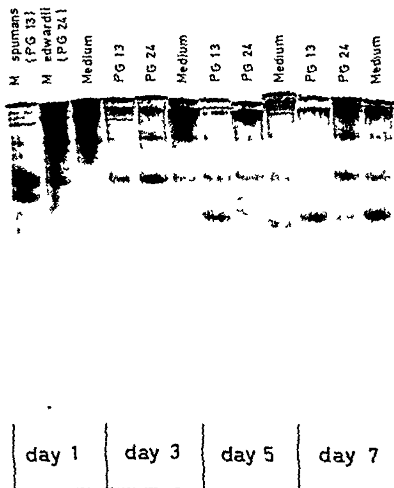


Fig 4 Electrophoretic patterns of protein (0 5 mg per gel) obtained by centrifugation at day 1 3 5 and 7 of *M spumans* (PG 13) culture *M edwardii* (PG 24) culture and uninoculated medium. Culture medium containing horse serum B

within the range of 25 to 50 μg throughout the period of incubation Using this medium the protein sediment was not either significantly larger in the inoculated portions

c Comparison of the Electrophoretic Patterns of Protein Harvested at Days 1 3 5 and 7

In *Fig 3* are shown the electrophoretic patterns of protein harvested from the medium containing horse serum A The protein from the uninoculated medium gave two weak and one strong band at day 1 At day 3 ten bands were present and this pattern grew a little more intense at days 5 and 7 The patterns of protein of the cultures did change with the time of incubation in such a way that they became similar to the pattern of protein from the uninoculated medium

The patterns of protein from the inactivated medium showed the same characteristics as just mentioned but patterns of protein from medium containing horse serum B showed an even closer similarity between uninoculated and inoculated cultures (Fig 4)

In Fig 5 are shown the electrophoretic patterns of the protein harvested from medi

um containing PPLO serum fraction Protein harvested after 1 day gave no pattern neither from cultures nor from uninoculated medium On the 3rd day, no bands were found from the uninoculated medium but on day 5 and 7 a weak band was seen at a distance of 1 5 cm from the application point The patterns of protein from the cultures did not change between day 3 and 7

d Comparison of Patterns of Protein Harvested from Different Media

No differences in protein patterns of the medium containing horse serum A and the corresponding inactivated medium were found On the other hand there was a clear difference between patterns of protein from media containing horse serum A and B respectively (Fig 3 and 4)

The patterns of *M spumans* (PG 13) and *M edwardii* (PG 24) grown in media containing horse serum possessed some bands in the upper centimeter of the gels bands which did not appear in the patterns of the corresponding strains grown in medium containing PPLO serum fraction Since these bands were found also in the patterns of protein from the uninoculated media containing horse serum they must be ascribed to the latter component in the medium

Experiment 2 Electrophoresis of 53 Mycoplasma Strains

a Identification of Strains

Prior to the comparative electrophoresis study the 50 isolated mycoplasma strains had been identified serologically Twenty four strains were identified as *M canis* using indirect immunofluorescence Fifteen strains were identified as *M edwardii* by the immunofluorescence method as well as by growth inhibition The remaining 11 strains were classified as *M spumans* On the basis of immunofluorescence and growth inhibition studies the latter strains were placed in two serological subgroups Serogroup I and II each containing 4 and 7 strains respectively I was serologically identical with the type strain of *M spumans* (PG 13)

b Culturing

On the basis of the studies on the influence of medium composition and time of cultivation it was chosen to cultivate the mycoplasma strains in medium supplemented with 2 per cent PPLO serum fraction and to incubate all cultures for 3 days at 37° C

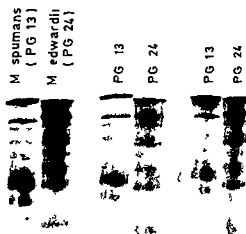


Fig 5 Electrophoretic patterns of protein (0 5 mg per gel) obtained by centrifugation at day 3 5 and 7 of *M spu*[illegible] (PG 13) and *M edwardi*[illegible] (PG 24) cultu[illegible] obtained at day 1 gave no patterns Pr[illegible] [illegible]oculated control me-dium gave a [illegible] 5 and 7 only Culture medium con[illegible] [illegible] serum fraction was used



Fig 6 The electrophoretic pattern of six *M canis* strains 0 5 mg protein applied per gel

c Comparison of Electrophoretic Patterns within Species

Generally the electrophoretic separation of protein from the mycoplasma strains resulted in 20 25 bands spread throughout the total length of the gel (approximately 6 cm) The bands were usually arranged more closely together and were more heavy in the upper part of the gels In the lower part they were weaker and more diffuse In spite of the fact that the time of electrophoresis was exactly 2 hours there could be small variations from gel to gel in the migration of the protein bands As a result the patterns could be either slightly compressed or more or less extended When compared the gels were therefore kept close together and analysed carefully from above and downwards

M canis Electrophoresis of protein from *M canis* strains resulted in patterns of about 20 bands (Fig 6) In some strains certain bands could vary in intensity and in others be totally lacking On the whole the strains gave a pattern of a uniform structure in all gels

M edwardii The pattern consisted of about 25 bands some of which varied in intensity and occasionally might be totally lacking

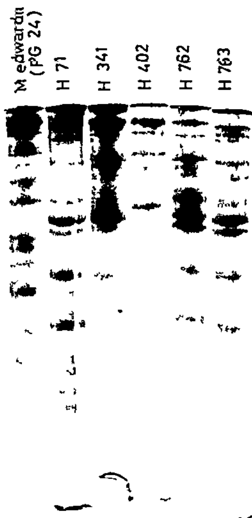


Fig 7 The electrophoretic pattern of six *M edwardii* strains 0 5 mg protein applied per gel

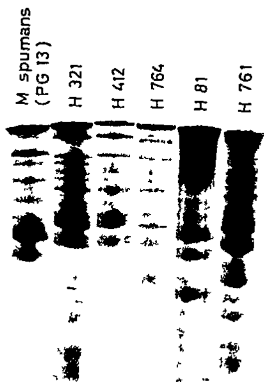


Fig. 8. The electrophoretic pattern of six *M. spumans* strains. Three belong to serogroup I (PG 13, H 321 and H 412) and three belong to serogroup II (H 764, H 81 and H 761). 0.5 mg protein applied per gel.

On the whole the pattern was characteristic and uniform from gel to gel (Fig. 7).

M. spumans. About 22 bands were found in the patterns of the *M. spumans* strains. Structural differences between the pattern of strains of serogroup I and of serogroup II could not be demonstrated. However, there was a minor variation in the upper 5 or 6 bands, in which respect the pattern of *M. spumans* (PG 13) seemed to differ from those of the other *M. spumans* strains (Fig. 8).

d. Comparison of Electrophoretic Patterns of Different Species

By comparison of the patterns of *M. spumans* strains and those of *M. canis* and *M. edwardii*, differences were easily recognized. These inter species differences superseded by far the observed minute variations between strains within each species (Fig. 6, 7 and 8). On the face of it, the *M. canis* and *M. edwardii* strains for an immediate observation showed patterns which were difficult to distinguish from each other. But there were differences in that *M. edwardii* possessed bands in the upper centimeter of the gel which *M. canis* did not possess. Furthermore in the lower part of the *M. canis* pattern there were two bands which appeared separated in the *M. edwardii* pattern (Fig. 7 and 8).

A blind trial was carried out in which 24 coded and mixed gels of *M. edwardii* and *M. canis* could be sorted out without any mistake.

DISCUSSION

Razin et al. (6) found that components of protein nature could be sedimented from media containing horse serum after 48 hours incubation at 37° C. They also found that such sediment from media containing PPLO serum fraction was very small. The methodological studies in this work confirm these observations.

Besides a quantitative difference between protein sedimented from cultures containing horse serum and cultures containing PPLO serum fraction, a qualitative difference seemed to be in evidence. This is demonstrated by the fact that it is only when a very weak band is obtained after electrophoresis of 0.5 mg of protein sedimented from uninoculated culture containing PPLO serum fraction that a distinct pattern is produced by electrophoresis of 0.5 mg protein sedimented from uninoculated culture containing horse serum.

By electrophoretic analysis of mycoplasma

strains isolated from dogs, *Armstrong & Yu* (1) who used growth medium containing horse serum found that the pattern changed during the period of incubation This is in agreement with findings in the present work if cultivation media containing 20 per cent horse serum were used, whereas the pattern did not change during the period of incuba tion if horse serum was replaced by 2 per cent PPLO serum fraction

The comparative studies demonstrated that electrophoretic separation of mycoplasma proteins resulted in a pattern exhibiting great uniformity between strains within species However, the intensity of some bands might vary and a few might even be lacking Strains belonging to two serogroups within *M spumans* showed uniform patterns which support the classification of the strains into one spe cies However, the pattern of PG 13 differed from the recently isolated *M spumans* strains, thus suggesting that there may be differences between the patterns of laboratory strains and those of fresh isolates *Razin* (4) found variations in the pattern of *M hominis* strains and ascribed this to a simultaneous antigenic variation as found in growth inhibition stu dies, In the present study, the serologic differences of the two subgroups of *M spumans* did not correlate with a difference in the electrophoretic patterns

On the basis of the above studies it can be concluded that growth media containing PPLO serum fraction is most suitable for cultivation of mycoplasma strains for electro phoretic examination even though the growth is a little delayed and inferior to that of media containing horse serum

It can also be concluded that electrophoretic analyses of mycoplasma proteins may aid in the classification of mycoplasmas isolated from dogs, as the patterns of 24 *M canis* 15 *M edwardii* and 11 *M spumans* strains can be differentiated from each other al though minor intra species variations do exist

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FATE OF EXOGENOUS DNA IN *NEISSERIA MENINGITIDIS*

1 Preparation of ^{15}N donor DNA and its Fate in Transformation

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A method is presented for the isolation of ^{15}N^{3}H DNA from *Neisseria meningitidis* The ^{15}N DNA separates from ordinary ^{14}N DNA of *N meningitidis* when analysed in analytical density centrifugation corresponding to a difference in buoyant density of approximately 0 013 g/cm^3 The specific density of regular *N meningitidis* DNA is found to be 1 7105 g/cm^3 Analysis by preparative density centrifugation of cell extracts of *N meningitidis* transformed with ^{15}N^{3}H DNA shows that the radioactivity taken up during transformation assumes a specific density very close to that of the recipient DNA The absence of radioactivity corresponding to that of the donor DNA in these cell extracts supports the notion that *N meningitidis* lacks a DNase insensitive site outside the membrane and supports the hypothesis of a concomitant uptake and integration of homologous exogenous DNA in *N meningitidis* transformation

The integration of genetic material is an intriguing biological process and the advantage of the transformation system is the possibility of characterization and modification of donor DNA In clarifying the transformation mechanism in *Neisseria meningitidis* several techniques have been applied, and a certain picture has emerged Genetic evidence indicates that in *N meningitidis* transformation proceeds sequentially in an order corresponding to that of the chromosome of the recipient cell (13) and that the DNA enters the cell via the point in the membrane to which the replication fork is attached by a mechanism of homology Physical evidence indicates that the amount of exogenous DNA taken up by *N meningitidis* under normal circumstances that is during a normal transformation procedure increases with time and is proportional to the transformation efficiency (15) Attempts to isolate single stranded DNA during the transformation process have been altogether negative (15) indicating that if there exists a single stranded intermediate stage between DNA uptake and integration the single stranded DNA is removed before it can be detected by physical means Similarly it has not been possible to detect any radioactive nucleotides which might be expected to originate from the strand not used for integration The current hypothesis is that in competent *Bacillus subtilis* *Streptococcus pneumoniae* as well as in *Haemophilus influenzae* the transformation systems most thoroughly studied the DNA penetrates to a location between the cell wall and the membrane where it is inaccessible for the DNase added to interrupt the transformation (1 17 (9) In the case of the pneumococci there even seems to be an immediate splitting of the

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double-stranded DNA into one single strand plus dialyzable oligonucleotides and inorganic phosphate (18) regardless of the origin of the DNA added (19) All genetic and physical evidence fits with the supposition that *N meningitidis* may lack this DNase insensitive site outside the membrane suggested for the other transformable species and in this case the irreversible uptake of exogenous DNA would directly reflect the DNA that has penetrated the membrane If this is true one would expect the donor DNA in *N meningitidis* transformation to undergo recombination concomitantly with the uptake to DNase insensitivity

The present report presents a method for the preparation of ^{15}N DNA from *N meningitidis*, and the use of such DNA in transformation It also describes the reextraction of the nucleic acids after transformation, and analysis of their characteristics by density gradient centrifugation

MATERIALS AND METHODS

Many of the media and manipulations used in the present study are standard procedures in this laboratory and have thus been previously described (13, 14, 15) Some procedures which have been modified, and some manipulations pertinent to the present work are listed below

Bacterial strains The strain used for the preparation of *E coli* ^{15}N extract was *E coli* K12 T71 (10) The mutants of *N meningitidis* used were derived from the wild type strain M1 (13) DNA was prepared from strain M1 Str r, a single step high level resistant mutant (12) As recipient in transformation experiments was used the mutant *M1 his arg cp⁺* Genetic competence has been indicated by the symbol *cp⁺*

Preparation of ^{15}N E coli *extract* The cells were grown on the minimal medium described by *Davis* &*Mingioli* (7) where the $(NH_4)_2SO_4$ had been replaced by $^{15}NH_4Cl$ The plates were incubated at 37°C overnight, the cells harvested in sterile SSC (0 14 M NaCl 0 015 M Na_3citrate, pH 7 4) The cell suspension was frozen and thawed whereupon batches of 3 ml were treated for 3 min in the MSE Ultrasonic Power Unit The extract thus obtained was incubated with gentle shaking at 37°C with 1 mg pronase (Calbiochem) per ml for 75 min, and finally filtered through Millipore HA 0 45 μ The extract, when diluted 1/100 showed an absorbancy of 0 345 when measured at 260 nm in the Beckman DB Spectrophotometer

Preparation of ^{3}H labelled, ^{15}N Neisseria meningitidis *DNA* The medium contained per litre K_2HPO_4 – 7 0 g, KH_2PO_4 – 2 0 g NA$_2$glutamate – 1 0 g Na_2SO_4 – 50 mg, $N_2S_2O_3$ – 50 mg $^{15}NH_4Cl$ – 1 0 g, Bacto agar (Difco) – 15 0 g, $MgCl_2$ – 0 1 mg $CaCl_2$ – 0 05 mg $MnCl_2$ – 0 01 mg, K lactate – 11 0 ml of a 50 per cent solution, Glycerol – 0 5 ml, *E coli* ^{15}N extract – 25 ml, Adenine–2–^{3}H – 10 mCi (4 3 Ci/mmol) The

upon the plates were incubated for another 24 h and harvested again The DNA was extracted by the procedure described earlier (15) ensuring DNA of a relatively high purity

Transformation procedure In order to increase the transformation efficiency, the treatment of the recipient cells prior to the exposure to the exogenous DNA has been modified The cells were inoculated into BH (Difco) broth from an overnight blood agar culture to give an absorbancy of 0 200

vested by centrifuging at 2300 *x* g for 10 min at room temperature and suspended in an equal volume of 2 5 per cent NaCl in the basal salts (per litre NaCl – 25 g, K_2HPO_4 – 7 0 g KH_2PO_4 – 3 0 g, NH_4Cl – 1 0 g, glutamic acid – 1 0 g Na_2SO_4 – 50 mg, $Na_2S_2O_3$ – 50 mg, MgCl – 0 1 mg, $CaCl_2$ – 0 05 mg, $MnCl_2$ – 0 01 mg), and this suspension was left at room temperature for 20 min when it was centrifuged at 2300 x g for another 10 min at room temperature The pellet was resuspended in HIB (Difco) broth and immediately mixed with the prewarmed (37°C) HIB – CaCl to which the DNA was added at the same time, giving a final concentration of 3 5 μg per ml The transformation was allowed to take place at 37°C with gentle shaking, usually for 30 min, and the transformation was terminated by the addition of DNase to give a final concentration of 50 μg per ml The incubation was continued in the shaker for another 5 min when the whole mixture was rapidly cooled

Reextraction of the nuclei acids after transformation The entire transformation mixture was centrifuged in the cold at 12500 x g for 30 min, and the cells thus harvested were washed by resuspension and centrifugation twice in HIB containing 50 μg DNase per ml, once in pure HIB, twice in SSC The cells were lysed as described earlier (15) In the present work the nucleic acids (either as crude extract, deproteinized and ethanol precipitated extract or RNase treated extract) were pressed ten

times through a No 20 hypodermic needle in order to decrease the viscosity of the extract prior to the density gradient centrifugation (27)

Density gradient centrifugation Preparative density gradient centrifugation was carried out in a total volume of 3 ml The stock solution of CsCl contained 1 1713 g Cs Cl per ml in standard SSC buffer (pH 7 4) 2 4 ml of this stock was mixed with 0 6 ml of the extract containing the nucleic acids This gives a density close to 1 710 g/cm³ The samples were placed in polyallomer tubes in the SW 39 L rotor or the SW 50 1 rotor of the Spinco Model L centrifuge and were spun for 70 h at 35000 RPM at 5°C The samples were collected as described earlier (15) by puncturing the bottom of the tubes In these experiments the fractions in the region of interest were approximately 40 μl The fractions were diluted with 0 5 ml distilled water, measured at 260 nm in the Beckman DB Spectrophotometer, transferred to polyethylene counting vials (Packard) where 10 ml Insta gel (Packard Instrument Co, Inc) was added The counting was performed in a Packard Tri Carb liquid scintillation spectrometer

The analytical density gradient centrifugations were carried out by dr *Terje Christensen,* Department of Biochemistry, University of Oslo CsCl was dissolved in distilled water (0 972 g CsCl per ml H_2O) and filtered once through Millipore HA 0 45 μ Approximately 0 7 ml of the CsCl solution containing about 1 μg of each of the two DNAs to be compared (the mixture was calculated to have a density close to 1 710 g/cm³) was placed in a plastic Kel F centerpiece and centrifuged in a Spinco Model E analytical centrifuge at 44500 RPM for 24 h Registration was carried out by means of a photoelectric scanner The DNA was not sheared prior to the analytical density gradient centrifugation

Chemicals The ¹ NH_4Cl 95 per cent was obtained from Isomet Corporation Oakland New Jersey 07436 U S A The CsCl was a product of Koch Light Laboratories Ltd Colnbrook Bucks England Adenine 2 ³H was purchased from the Radiochemical Centre Amersham Bucks England Potassium Lactate was a product of BDH Chemicals Ltd, Poole England a solution of approximately *50 per cent*

RESULTS

Characteristics of the donor DNA Growth of *N meningitidis* on the ^{14}N containing medium described in the section on methods ensured DNA preparations of sufficient quality for the present purpose They regularly contained some RNA the proportion of DNA RNA ranging from 4 1 to 10 1 The

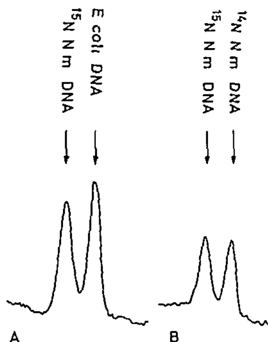


Fig 1 A A microdensitometer tracing showing the separation of ^{15}N *N meningitidis* DNA and *E coli* DNA The position of the peaks corresponds to a difference in buoyant density of 0 0133 g/cm³
B A microdensitometer tracing sl cwing the separation of ^{14}N and ^{15}N DNA from *N meningitidis* The distance between the peaks corresponds to a *difference in buoyant density of 0 0128 g/cm³*
The tracing was performed on photographs taken after 24 h centrifugation at 44500 RPM in the Spinco Model E Analytical Centrifuge

specific activity of the various DNA preparations was in the range of 1 1 × 10⁴ to 1 7 × 10⁴ cmp per μg nucleic acid

Results from analytical density gradient centrifugations can be seen in Fig 1 Fig 1 A is the result of a run where the ^{15}N *N meningitidis* DNA has been mixed with *E coli* DNA as a reference On the basis of the specific density of *E coli* DNA which is said to be 1 710 g/cm³ (23) the specific density of the ^{15}N *N meningitidis* DNA is calculated to be 1 7233 g/cm³ Fig 1 B is the result of a run on a mixture of ^{15}N *N meningitidis* DNA and ordinary ^{14}N *N meningitidis* DNA In this run the reference is the newly calculated ^{15}N *N meningitidis* DNA of 1 7233 g/cm³, and the specific density of the ^{14}N *N meningitidis* DNA is then calculated to be *1 7105* g/cm³

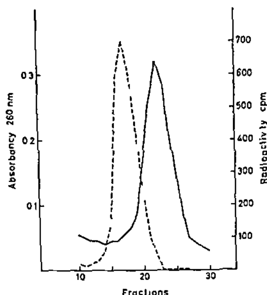


Fig 2 Separation of $^{15}N^{3}H$ DNA (donor DNA) and ^{14}N DNA (recipient DNA) from *N meningitidis* in the preparative ultracentrifuge run for 70 h at 35000 RPM at 5°C The $^{15}N^{3}H$ DNA which is recorded as *cpm* (dotted line) has been added to a lysed extract of ^{14}N grown *N meningitidis* cells whose DNA is recorded by absorbancy at 260 nm in the Beckman DB Spectrophotometer (solid line)

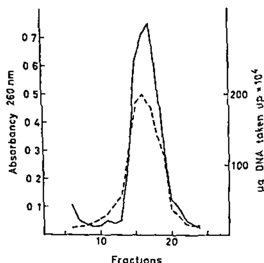


Fig 3 Extraction of labelled material from *N meningitidis cp*$^{+}$ which has been transformed with $^{15}N^{3}H$ DNA Density gradient centrifugation (pH 7 4) of cells exposed to $^{15}N^{3}H$ DNA (specific activity 1 39 × 10^{4} cpm/µg NA) for 30 min Transformation frequency (str r/str s) was 1 23 × 10^{-3} Absorbancy = solid line Radioactivity -- dotted line

The calculations have been performed by dr *Terje Christensen*

The difference in the buoyant densities of the ^{15}N and the ^{14}N *N meningitidis* DNA is also apparent in the preparative ultracentrifungation density gradient, as seen in Fig 2 The ^{15}N DNA separates by four fractions from the ordinary ^{14}N DNA when measured by radioactivity and absorbancy at 260 nm respectively The proportion of ^{15}N DNA to ^{14}N DNA (cell extract) has been designed to mimick the proportion of donor DNA to recipient DNA in an actual transformation experiment

Fate of ^{15}N donor DNA in transformation of Neisseria meningitidis Fig 3 shows a typical example of the fate of ^{15}N donor DNA in an ordinary transformation of *N meningitidis, cp*$^{+}$ The transformation frequency in this particular experiment is fairly high, 1 2 × 10^{-3}, as is the uptake of exogenous DNA, which is calculated to be 9 6 × 10^{-4} µg DNA taken up per µg total DNA as measured by A_{260} This is in accordance with earlier findings of a proportionality between DNA taken up and the transformation efficiency (15) The particular density gradient centrifugation recorded in Fig 3 is performed on a deproteinized, ethanol precipitated extract Density gradient centrifugations on crude extracts sometimes show spurious bands (15, 19) The radioactivity taken up by the cells is found in one peak with an average density very close to, but slightly heavier than, that of the normal, double stranded DNA of the recipient cells as measured by A_{260} Normalization of the curves by a probit transformation (14) also showed this tendency

DISCUSSION

Neisseria meningitidis is unable to utilize exogenously supplied thymine (16) to an extent which might make it possible to use bromuracil as the means of increasing the specific density of the DNA Attempts to grow *N meningitidis* on media where the H_2O had been replaced by D_2O were altogether negative $^{15}NH_4Cl$ as the sole source of nitrogen was

then chosen as a practicable means of increasing the specific density of the meningococcus DNA (22) In the hands of *Meselson & Stahl* (22) the difference of buoyant density between *E coli* DNA from cells grown on ^{14}N medium (which they found to be 1 710 g/cm^3) and *E coli* DNA from cells grown on $^{15}NH_4Cl$ of a purity of 96 5 per cent corresponded to 0 014 g/cm^3 In their system which is well known this was sufficient to separate heavy light and hybrid DNA

E coli grows very well on a completely synthetic medium and it was sufficient for *Meselson & Stahl* to replace the $^{14}NH_4Cl$ with $^{15}NH_4Cl$ to obtain their results *N meningitidis* is also able to grow on completely defined medium with glutamic acid as the sole source of carbon and energy (11) but the growth yield is fairly scanty and in order to obtain ^{15}N^{3}H DNA in quantities large enough to perform large scale transformation experiments within reasonable expences it was desirable to increase the cell yield Addition of arginine cysteine and glycine to the synthetic medium will increase the cell harvest considerably (5) and a similar increase may be obtained by addition of commercially obtained casamino acids It was found that *N meningitidis* responded well to increasing amounts of *E coli* extracts and an optimum was found to give sufficient growth without interfering with the uptake of ^{3}H adenine

Results from analytical density centrifugations (as shown in Fig 1) show a separation between the heavy ^{15}N DNA and the normal *N meningitidis* DNA corresponding to a difference in buoyant density of close to 0 013 g/cm^3 Using *E coli* DNA as a reference in order to calculate the specific densities of both ^{15}N *N meningitidis* DNA and ordinary *N meningitidis* DNA gives the densities of 1 7233 g/cm^3 and 1 7105 g/cm^3 respectively The base composition of DNA from *N meningitidis* strain Ne 15 was determined by *Catlin & Cunningham* (4) to have a G + C content of 51 3 per cent and later *Marmur & Doty* (21) found it to be 51 5 per cent as determined by thermal denaturation *Schildkraut et al* (23) list the specific density of the same strain of *N meningitidis* Ne 15 as determined by buoyant density in CsCl to be 1 703 g/cm^3 referring to the literature giving the G + C content of 50–51 per cent In the same list these authors report the specific density of *E coli* DNA to be 1 710 g/cm^3 and the G + C content to be 50–51 per cent There thus seems to be a slight descrepancy between the specific density of *N meningitidis* as determined by buoyant density and the G + C content and one should expect the specific density of *N meningitidis* to be 1 710 g/cm^3 or even slightly higher The present finding of a specific density of 1 7105 g/cm^3 for *N meningitidis* DNA is more in accordance with the G + C content as reported in the literature although it must be kept in mind that the DNA originates from a different strain of *N meningitidis* Ml of sero-group B (14)

In the other transformable species thoroughly studied transformation seems to be a very efficient process In a phenotypically competent population of *Str pneumoniae* it is reported that all cells in the population take up exogenous DNA (9) and the transformation efficiency is of the order of 10^{-1} In *H influenzae* the transformation efficiency is of the order of 10 (26) and similar frequences are reported for *B subtilis* where approximately 2–10 per cent of the competent population takes up exogenous DNA (2 24) *N meningitidis* is much less efficient in transformation and a transformation efficiency approaching 10^{-3} has been considered good The treatment of the recipient cells prior to the exposure to exogenous DNA introduced in the present study (see materials and methods) seems to increase the transformation frequency by a factor of 2 3 The reason for this increase is not clear but it may partially be due to a removal of extracellular DNA which always seems to be present in *N meningitidis* populations and which is known to transform competent cultures of *N meningitidis* (3 20) Sodium chloride is reported to enhance transformation in *Bacillus amyloliquefaciens* by a factor of 2 5 and the optimal concentration of NaCl is 0 6–0 7 M (6) A similar enhancement is found in *Bacillus*

licheniformis with an optimum at 0 4M NaCl (25) The molarity of NaCl used in the present treatment of *N meningitidis* is 0 42 M

Because of the small DNA uptake in *N meningitidis* transformation it is necessary to load the tubes for preparative density gradient centrifugation with cell extracts representing from 75 μg to 150 μg DNA In order to achieve separation of DNA with different buoyant densities it is essential to decrease the viscosity When extracts of *B subtilis* were passed 7 times through No 27 hypodermic needles *Takahashi* & *Ikeda* (27) obtained DNA pieces of a highly uniform size with an average molecular weight of 9×10^6 daltons In our system ^{15}N DNA separates well from the bulk DNA (Fig 2) when the cell extracts are pressed 10 times through a No 20 hypodermic needle

The present experiments indicate that DNA taken up by competent *N meningitidis* cells during transformation is immediately integrated into the recipient chromosome, since the radioactivity of the $^{15}N^3H$ donor DNA is found in one peak with a specific density which is very close to that of the recipient DNA The density gradient centrifugations reveal no peaks corresponding to that of the original ^{15}N donor DNA Thus if uptake and integration are separate steps in the meningococcus no pool of donor DNA is allowed to accumulate, suggesting at least a rapid integration of DNA slowly taken up

The peak of radioactivity taken up by the recipient cells during transformation is not completely identical to that of the bulk DNA but shows a slight shift towards the heavier side This is of course to be expected since it is necessarily of hybrid origin The difference of the two peaks is however very small rather less than one fraction Very recently *Dubnau* & *Cirigliano* (8) report from a very similar type of experiments in *B subtilis* that in that system where they find the average size of the single stranded donor segment incorporated during transformation to be $2\ 8 \times 10^6$ daltons the donor recipient hybrid DNA had to be sheared to an average molecular weight of $6\ 45 \times 10^6$ in order to obtain a pure hybrid position in the CsCl density gradient The shearing force applied in the present experiments has hardly chopped the donor recipient complex down to an average molecular weight anywhere near 6×10^6 But then, at present nothing is known about the average size and distribution of single stranded *N meningitidis* DNA segments incorporated They may well be different from those of *B subtilis*, and this question may be well worth pursuing

Previous studies with alkaline density gradient centrifugations revealed no accumulation of single stranded DNA (15) Although the present density gradient centrifugations are neutral (pH 7 4) single strands of donor DNA would be expected to reveal themselves with a specific density greater than that of the double stranded donor DNA Again, no such accumulation is indicated in the present experiments The present results are in accordance with the hypothesis of a concomitant uptake and integration of exogenous DNA is *Neisseria meningitidis* transformation

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FATE OF EXOGENOUS DNA IN *NEISSERIA MENINGITIDIS*

2 Influence of Acriflavin and Ethidium Bromide and Significance of Genetic Competence on the Fate of ¹⁵N DNA

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Density gradient centrifugations of cell extracts from *Neisseria meningitidis* transformed by $^{15}N^{3}H$ DNA in the presence of acriflavin or ethidium bromide show that in spite of a substantial reduction of viable transformants in the presence of these drugs, the radioactivity taken up assumes a specific density indistinguishable from that of the DNA of the recipient cells Thus DNA uptake and integration are inseparable steps in *N meningitidis* The genetically incompetent (*cp*) variant of *N meningitidis* is unable to integrate exogenous DNA into its chromosome Furthermore it does not take up exogenous DNA to DNase insensitivity neither homologous nor heterologous DNA Exogenous DNA is only taken up by genetically competent (*cp*⁺) *N meningitidis* if of homologous origin and when it is integrated into the recipient chromosome in a transformation event

Analysis by preparative density centrifugation of cell extracts of *Neisseria meningitidis* transformed with $^{15}N^{3}H$ DNA supports the former notion that uptake and integration of exogenous homologous DNA are concomitant in *N meningitidis* transformation The transformation mechanism in *N meningitidis* is thus very different from that in the other systems which have been thoroughly studied from this point of view *Bacillus subtilis*, *Streptococcus pneumoniae* and *Haemophilus influenzae* (6) A common feature of all these systems is a rapid uptake of relatively large amounts of exogenous DNA, homologous as well as heterologous followed by an integration if the DNA is of homologous origin This integration may take place more or less rapidly and it is possible to follow the process by physical means

When *B subtilis* is exposed to homologous DNA in the presence of acriflavin the irreversible uptake of DNA into the competent cells is not inhibited, but the transformation is reduced to a negligible value (2) By using heavy, bromuracil labelled DNA it could be shown that the DNA taken up in the presence of acriflavin is never used for recombination, since it remains as double stranded donor DNA (2) Transformation in *N meningitidis* in the presence of acriflavin, as well as of ethidium bromide, revealed that with increasing concentrations of these drugs came a marked reduction in the DNA uptake (contrary to the situation in *B subtilis*), a progressive decrease in the overall cell population as well as a reduced but still notable, transformation frequency (11) The density gradient profiles of the DNA irreversibly bound by *N meningitidis* in the presence of these two DNA—intercalating drugs were somewhat distorted, a fact that might be interpreted as supporting a two phase uptake—recombina

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tion process also in *N meningitidis* Since the use of acriflavin and heavy donor DNA served to distinguish the two phases, DNA uptake and DNA integration, in *B subtilis* in such a highly illustrative fashion it was tempting to employ the same technique in the meningococcal system

Another point in which *N meningitidis* differs from the other transformable species is in regard to competence In order to be transformed the meningococcus must be genetically competent (cp^{+}) and this genetic competence may be irreversibly lost, converting the cells which lose it, and their descendants into genetically incompetent (*cp*) variants (14) However, when genetically competent, *N meningitidis* is phenotypically competent during the entire growth period (16), and thus very different from *B subtilis*, *Str pneumoniae* and *H influenzae* (1, 24, 21) that all show a characteristic peak of phenotypic competence at a certain stage in their growth cycle

There are two stages in the binding of DNA to any recipient cell the reversible binding and the irreversible DNase insentive binding Regarding the reversible binding to the *N meningitidis* cell surface this has been shown to be unspecific in the sense that any double stranded DNA of proper molecular weight is bound (15) In contrast the irreversible binding of DNA to *N meningitidis* seems to be limited to homologous DNA Of consequence is the fact that any unspecifically bound DNA inhibits transformation with genetically marked homologous DNA the extent of inhibition depending upon the source of the DNA The most efficient inhibitor is unmarked homologous DNA presumably because it is also irreversibly bound (13) The irreversible binding seems largely limited to cells of the *cp* genotype although isopycnic centrifugation of *cp N meningitidis* cells exposed to radioactively labelled homologous DNA did show a very slight accumulation of radioactivity corresponding to double stranded DNA It is of importance to clarify this point, since it may augment the understanding of the true activity of the cp factor (12) By the use of ^{15}N donor DNA it should be possible to clarify whether the incompetent variants of *N meningitidis*, if they really do take up exogenous DNA are able to integrate this DNA into their chromosomes In this way it should be possible to reveal whether the difference between the cp^{+} and the *cp* genotypes is of a qualitative rather than a quantitative nature Additionally, the present technique (6) pretreating the recipient cells prior to the exposure to the exogenous DNA which increases the transformation efficiency and thereby the uptake of exogenous DNA by the competent variant of *N meningitidis* may add to the sensitivity of the system

The fact that it seems that irreversible binding of DNA to *N meningitidis* may be limited to DNA of homologous origin is another feature that serves to distinguish *N meningitidis* from the other transformable species It would be interesting to reinvestigate this point by a more sensitive technique since such specificity might almost be taken to imply some extracellular re ognition mechanism in this system

The present investigation is an attempt to reveal the possible existence of a two-step uptake—integration process in *N meningitidis* and also an effort to elucidate genetic competence

MATERIALS AND METHODS

The ^{15}N ^{3}H DNA the transformation procedures as well as the reextraction of the nucleic acids and the ... been

The

study

Bacterial strains The ... in the present study is the mutant *M I his arg cp* and a variant of it having irreversibly lost its genetic competence *MI his arg cp* For the preparation of *E coli* ^{3}H DNA was used the strain *E coli K12 CR34 thr leu thi thy* which was kindly furnished by dr *Finn Haugli* at the University of Tromsø Norway The ^{3}H *N meningitidis* DNA was prepared from the mutant *MI str r* (6)

Preparation of DNA ^{3}H DNA from *N meningitidis* was prepared as described earlier (13) as was the ^{3}H DNA from *E coli* (5)

Administration of inhibitors When acriflavin or ethidium bromide was used in transformation the

TABLE 1 *Effect of Acriflavin and Ethidium Bromide on Transformation Efficiency, DNA Uptake and Viability of Cell Population*

Exp No	Inhibitor added	Transformation		Relative DNA Uptake		Col I units	
		T/E	per cent	$\mu g\ ^3H$ DNA/ μg total DNA	per cent	No	per cent
1	None	8.4×10^{-4}	100	16.3×10^{-4}	100	2.0×10^{8}	100
	Acriflavin	1.2×10^{-5}	1.4	1.9×10^{-4}	36	4.6×10^{7}	23
2	None	8.4×10^{-4}	100	16.3×10^{-4}	100	1.3×10^{8}	100
	E B	1.6×10^{-5}	1.9	6.4×10^{-4}	39	3.0×10	23

drugs were added to the prewarmed transformation mixture simultaneously with the recipient cells and the DNA Acriflavin was added to give a final concentration of 0.20 μg per ml ethidium bromide was added to give a final concentration of 6×10^{-6} M (11)

Chemicals Ethidium bromide (2 7-diamino-10 ethyl 9 phenyl phenanthridium bromide) was a product of Calbiochem Inc, Los Angeles California Acriflavin (a mixture of 2 8 (3 6) diamino 10 ethylacridinum chloride and 2 8 (3 6) diaminoacridine) was from Sigma Chemical Company, St Louis Missouri Thymine (methyl) H^3) was purchased from the Radiochemical Centre Amersham Bucks England

RESULTS

Influence of inhibitors on the fate of ^{15}N *donor DNA* The effect of acriflavin and ethidium bromide on the transformation frequency, the uptake of $^{15}N^3H$ labelled str r DNA and the overall cell population is shown in Table 1 The results listed in this table are in agreement with similar results obtained with ordinary $^{14}N^3H$ str r DNA (11) The reduction in transformation frequency in the presence of *ethidium bromide* (6×10^{-6} M) is practically identical whereas the reduction of the transformation frequency in the presence of 0.20 μg *acriflavin* is more pronounced in the present experiments This may not be of any significance however, since the actual reduction in transformation frequency may vary from one experiment to the other A common feature with both these DNA intercalating drugs seems to be that the reduction

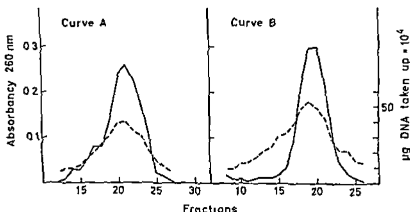


Fig 1 Effect of acriflavin (A) and ethidium bromide (B) on the uptake and fate of transforming $^{15}N^3H$ DNA during transformation of *N meningitidis* CsCl density gradients (pH 7.4) of extracts from cells (*MI arg his cp*$^{+}$) transformed with $^{15}N^3H$ labelled str r DNA for 30 min
Absorbancy solid line (recipient DNA) Radioactivity – dotted line (donor DNA given as $\mu g \times 10^4$)

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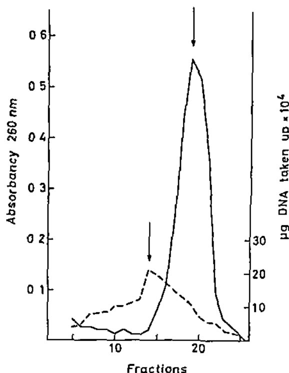


Fig 2 Fate of $^{15}N^{3}H$ DNA in non transformed *N meningitidis* cells Density gradient (pH 7 4) of extract from cells (*MI arg his cp*) exposed to $^{15}N^{3}H$ DNA from *MI str r* (specific activity 1 39 × 10^4 cpm/μg NA) for 30 min
Absorbancy – solid line (cell DNA) Radioactivity – dotted line (donor DNA given as μg × 10^4)

in transformation frequency is more pronounced than the reduction in the overall cell population This may reflect the metabolic state of the cells that are engaged in an actual transformation event Also the real DNA uptake is greater than what should be expected from the number of viable transformants This similarly is in agreement with earlier findings (11)

Fig 1 (A and B) shows that the main part of the radioactivity taken up by the recipient cells in the presence of acriflavin and ethidium bromide is found in a peak corresponding to that of the normal DNA of the recipient cells as recorded by the absorbancy measured at 260 nm in the Beckman DB Spectrophotometer Earlier findings in transformations with ordinary $^{14}N^{3}H$ labelled donor DNA showed a broadening of the radioactive peaks (11) of the DNA taken up in the presence of increasing concentrations of acriflavin as well as of ethidium bromide Such broadening of the radioactive DNA peak was distinct also at the concentrations used in the present experiments, and must thus be kept in mind when the present findings are evaluated

Fate of exogenous $^{15}N^{3}H$ DNA in non transformed N meningitidis Fig 2 shows a typical density gradient profile of cell extracts of *N meningitidis cp* exposed to $^{15}N^{3}H$ DNA under conditions leading to transformation in the *cp⁺* variant The radioactivity is found in a peak which is separated from the peak of the DNA of the *N meningitidis cp* by 4–5 fractions, corresponding to double stranded $^{15}N^{3}H$ DNA of *N meningitidis* (6) If one considers calculating the amount of exogenous DNA taken up by the *cp* related to the total amount of cell DNA it is logical to calculate from the maxima of the two peaks This indicates an uptake of 1 3 × 10^{-4} μg foreign DNA per μg total DNA This amount is much higher than that found earlier in the *cp* variant of *N meningitidis* (13)

Uptake of homologous ³H labelled DNA by non transformed N meningitidis Fig 3 shows typical profiles from isopycnic centrifugations of deproteinized and ethanol precipitated extracts of the genotypically incompetent variant of *N meningitidis* exposed to homologous ³H labelled DNA of a fairly high specific activity (2 7 × 10^4 cpm/μg NA) The exogenous DNA in Fig 3 A is biologically active and the transformation frequency in the control experiment with the competent variant as recipient was 1 3 × 10^{-3} The uptake of exogenous DNA by the non competent variant in this experiment, when calculated from the maximum of the DNA peak is 1 4 × 10^{-4} μg exogenous DNA per μg total DNA which is completely comparable to the amount found using the 'heavy' homologous DNA but again much higher than that found in previous experiments (11) The exogenous DNA in Fig 3 B had been degraded by treatment with DNase (50 μg/ml) for 10 min prior to the mixing with the *cp* cells the DNase being present throughout the experiment as well The control transformation

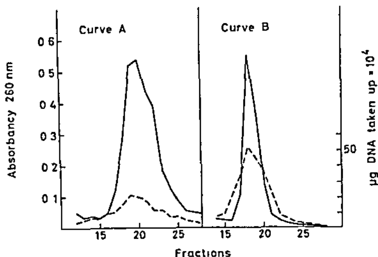


Fig 3 Density gradient profiles from cell extracts of non transformed *N meningitidis* (*M1 arg his cp*) exposed to homologous DNA [^{3}H DNA from *M1 str r*, specific activity 2 7 × 10⁴ cpm/μg NA]
A Exposure to DNA for 30 min when DNase was added
B DNase (50 μg/ml) plus DNA mixed 10 min prior to the addition of the *cp* cells, the DNase present throughout the incubation
Absorbancy – solid line Exogenous DNA (radioactivity) – dotted line

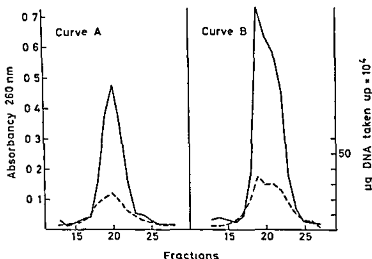


Fig 4 Density gradient profiles from cell extracts of competent *N meningitidis* (*M1 arg his cp*$^+$) exposed

with the competent variant under identical conditions showed a transformation frequency of 4×10^{-7}, which indicates a substantial reduction in biological activity This DNase-degraded DNA must in some way be attached to the cells, and it is distributed in the density gradient in the same fashion as the biologically active DNA The amount of nucleotides

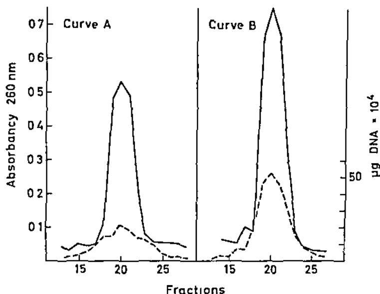


Fig 5 Density gradient profiles from cell extracts of *N meningitidis* incompetent in transformation (*Ml arg his cp*) exposed to ^{3}H labelled *E coli* DNA (specific activity 9.0 × 10^4 cpm/μg DNA)
A Cells incubated for 30 min with ^{3}H *E coli* DNA whereupon DNase was added
B Cells incubated with ^{3}H *E coli* DNA pretreated with DNase (50 μg/ml), the DNase present throughout the experiment
Absorbancy – solid line Foreign DNA – dotted line

attached to the cells related to the total DNA is again calculated from the maximum of the peaks, and in this experiment it equals 3.1 × 10^{-4} μg nucleotides per μg total DNA, a figure in fact even higher than that found with biologically active DNA

Fate of heterologous ^{3}H labelled DNA reexamined Since there is some evidence indicating that the treatment of the cells prior to the exposure to exogenous DNA (6) in some way affects the possibility of attachment of DNA as well as degraded DNA to the cells it was of some interest to see how such cells would react to DNA of heterologous origin The experiments of which typical examples are shown in Fig 4 and Fig 5, were carried out using ^{3}H labelled *E coli* DNA of high specific activity (9.0 × 10^4 cpm/μg DNA) The density gradient centrifugations were carried out on deproteinized ethanol precipitated extracts Fig 4 records the results from the competent variant of *N meningitidis*, (*cp*$^+$), when exposed to the ^{3}H labelled *E coli* DNA (Fig 4 A) and DNase-degraded ^{3}H labelled *E coli* DNA (Fig 4 B), and Fig 5 (A and B) similar recordings with the *cp* variant of *N meningitidis* under conditions similar to those of an ordinary transformation experiment In all four cases the radioactivity is distributed in peaks corresponding to that of the doublestranded DNA of the *N meningitidis* cells [which incidentally is another piece of evidence of the very great similarity of the specific density of *N meningitidis* DNA, which has been determined to be 1.7105 g/cm^3, to that of *E coli* DNA of 1.710 g/cm^3 (6) This is also indicated by their G + C content as recorded in the literature (4 18)] When calculating the amount of foreign DNA related to the amount of total DNA from the maxima of the peaks the values are 1.7 × 10^{-4} μg foreign DNA per μg total DNA (Fig 4 A) and 1.6 × 10^{-4} μg nucleotides per μg total DNA (Fig 4 B) when the competent variant of *N meningitidis* is exposed to DNA and degraded DNA from *E coli* respectively For the incompetent variant the values are 1.4 × 10^{-4} μg foreign DNA per μg total DNA (Fig 5 A) when the normal *E coli* DNA is used and 2.3 × 10^{-4}

μg nucleotides per μg total DNA (Fig 5 B) when the cells have been exposed to the degraded DNA Again it seems that more radioactivity is attached to the non competent variant cells when the DNA has been treated with DNase for a longer period of time

DISCUSSION

During normal transformation of *N meningitidis* there seems to be a strict proportionality between the DNA taken up and the transformation efficiency (15 13), and together with the evidence of the density gradient centrifugations of *N meningitidis* cells that had been transformed with $^{15}N^{3}H$ DNA this proportionality was fundamental for the [illegible]

taking place in the presence of acriflavin or ethidium bromide this proportionality is obscured When considering the facts of the somewhat reduced DNA uptake, the more marked reduction in the number of viable cells to the even more marked reduction of viable transformants it is plausible to visualize the process in the following manner The recipient cells the exogenous DNA and the inhibiting drug are mixed, and the transformation process starts in a normal way by the actively metabolizing, DNA replicating cells Although transformation in *N meningitidis* can take place in the absence of DNA replication (10) it is probable that it is normally taking place during active DNA replication (8) It is thus conceivable that the cells most actively DNA replicating in the heterogeneous recipient population are also most actively transforming, and thus more sensitive to the killing action of these DNA intercalating drugs These results thus emphasize another difference between *N meningitidis* and other transforming species Competent *B subtilis* are non dividing (19, 22), and competence in *H influenzae* also seems to be linked to a downshift in the cell metabolism (23) The density gradient profiles of cell extracts of *N meningitidis* transformed with $^{15}N^{3}H$ DNA in the presence of acriflavin or ethidium bromide did not distinguish between DNA uptake and DNA integration in this organism, since all radioactivity taken up by the cells in the presence of these drugs assumes a specific density similar to that of the recipient chromosome Thus these results represent another piece of evidence in support of the hypothesis of a concomitant uptake and integration of transforming DNA in *N meningitidis* Ethidium bromide is known to intercalate in the DNA structure, and it has been used as a means of isolating covalently closed circles of DNA from linear DNA structures, based on the fact that more ethidium bromide can be taken up by the linear structures than by the circular ones Thus the resulting specific density of linear DNA structures is decreased to a greater extent than the circular ones (20) The concentration of ethidium bromide used for that purpose (100–200 μg per ml in the CsCl preparation used for the density gradient centrifugations) is of quite another order of magnitude than that used in the present experiments where the concentration in the transformation mixture is 2 36 μg per ml Nevertheless it has been assured that this concentration has no noticeable effect on the specific density of the DNA

The results from experiments where the genetically incompetent variant of *N meningitidis* is exposed to $^{15}N^{3}H$ DNA of homologous origin clearly show that this variant is unable to integrate this 'heavy DNA into its chromosome Thus this must be interpreted as a definitive qualitative difference between the *cp*⁺ and the *cp* genotypes It has been shown that the origin as well as the direction of chromosome replication are different in the *cp*⁺ and the *cp* variants of *N meningitidis* (7, 9) It has been suggested that the replication point in *cp*⁺ variants is accessible for extracellular DNA in contrast to that of the *cp* variants (7) and it has been suggested that the genetic competence in fact is due to the insertion of a cytoplasmic particle in the chromosome Such a structure containing genetic information corresponding to a replicon might result in domination over the chromo-

somal replicon (9) Considering the transformation in *N meningitidis* being so closely related to DNA replication, one might speculate on whether the difference between these two genotypes is a difference of replication mechanisms which may (cp^+) or may not (cp) make use of available double stranded DNA in the DNA replication process

It was somewhat surprising, however, that the *cp* variant of *N meningitidis* in the present experiments showed such a high uptake of exogenous DNA, an apparent contradiction to the current hypothesis of simultaneous uptake and integration The reason for this increase in uptake may be the new treatment of the cells prior to the exposure to exogenous DNA (6) It has been shown that in growing cultures of *N meningitidis* there are large quantities of extracellular DNA, and this DNA can transform if the cells are of the competent genotype (3) or if competent and incompetent cells are growing in mixed culture (17) One can imagine that the increase in transformation frequency which is observed after centrifugation, resuspension in hypertonic salt solution and recentrifugation is partly due to removal of extracellular DNA and that the exogenous DNA added after this treatment may stick to the cells more or less unspecifically Results with the incompetent variant of *N meningitidis* being exposed to biologically active and to DNase degraded DNA show that this interpretation may be correct, since the amount of DNA taken up is more or less the same whether the DNA is biologically active or degraded—in the latter case rather more radioactivity is found in the cell extracts Thus one cannot speak of DNA taken up to a DNase insensitive site but rather the more the DNA is degraded the more radioactivity sticks to the cells

If the radioactive peaks found when the *cp* variant of *N meningitidis* is exposed to biologically active or degraded DNA really are due to unspecific attachment one should expect similar results when exposing the cells to DNA of heterologous origin and that is just the case *E coli* DNA of high specific activity sticks to the competent as well as to the incompetent variant of *N meningitidis* in amounts comparable to those found when the incompetent variant is exposed to homologous DNA

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IDENTIFICATION AND QUANTITATION OF PRECIPITINS AGAINST *PSEUDOMONAS AERUGINOSA* IN PATIENTS WITH CYSTIC FIBROSIS BY MEANS OF CROSSED IMMUNOELECTROPHORESIS WITH INTERMEDIATE GEL

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Crossed immunoelectrophoresis with intermediate gel was used in an attempt to identify and quantitate precipitins against *Pseudomonas aeruginosa* in sera from 33 patients with Cystic Fibrosis A standard antigen antibody system containing 55 precipitates was used as reference A total of 173 precipitins against Pseudomonas aeruginosa were found in 20 of the sera 56 per cent of the precipitins representing 30 different precipitin specificities could be identified and quantitated in relation to the reference system As much as 22 precipitins were found in one serum In one serum the simultaneous occurrence of 6 *Pseudomonas aeruginosa* antigens and 15 *Pseudomonas aeruginosa* precipitins were demonstrated Patients harbouring mucoid strains of *Pseudomonas aeruginosa* in the respiratory tract were found to produce significantly more precipitins than other groups of subjects and 23 out of the 30 different identified *Pseudomonas aeruginosa* precipitins were only found in this group of patients. Protective antibodies were not demonstrated It is suggested that the mucoid strains of *Pseudomonas aeruginosa* characteristic of patients with Cystic Fibrosis are selected in preference to the non mucoid strains by means of the patient s immune response The persistent infection and the multiple precipitins produced against the bacteria by many of the patients raise the question whether the local immune reaction could enhance the destructive lesions of the respiratory tract

Infections caused by *Pseudomonas aeruginosa* (Ps aeruginosa) have created increasing problems during the latest decade It is often difficult to distinguish between harmless colonization and infection causing damage to the

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tissues and it is difficult to combat such infections by means of antibiotics

Patients (pts) suffering from Cystic Fibrosis (C F) are known to contract frequent respiratory tract infections, and *Ps aeruginosa* are among the bacteria most frequently isolated from the sputum of these pts (21) Furthermore, *Ps aeruginosa* isolated from these pts (C F 's) are most often mucoid strains Such strains are rarely isolated from other pts (11, 12, 13, 14) Until now the only demonstrated differences between mucoid strains and non mucoid strains are apparently the amount of mucus produced and the composition of the mucus the mucus of the mucoid strains contains uronic acids which are not found in the mucus of the non mucoid strains Phagetyping, serotyping and pyocintyping show no differences between the two kinds of strains (11, 12, 14)

In recent years an increasing interest has been attached to investigations of the immune response to infections with *Ps aeruginosa* in order that pts who are definitely infected and not only colonized might be selected Consequently, the application of rather toxic antibiotics could be restricted and be used only in the treatment of pts suffering from *Ps aeruginosa* infection, besides possibly protective antibodies could be discovered and used as a basis for immunotherapy (1, 6, 7, 9, 10, 17, 24, 25 27) The immunological investigation of sera from pts infected with *Ps aeruginosa* is complicated because *Ps aeruginosa* can be divided into several serogroups (16, 20 23) and because one and the same pt can harbour *Ps aeruginosa* from different serogroups at the same time (11)

C F 's have been examined for the occurrence of antibodies against *Ps aeruginosa* by several investigators and several different methods have been used (8 11, 15, 19, 22) Up to 6 different antibodies against *Ps aeruginosa* have been found in a single serum (8, 11) and *Ps aeruginosa* agglutinins have been demonstrated in up to 100 per cent of sera from C F s the titres being strongest in pts harbouring *Ps aeruginosa* in the sputum (19) Precipitins against *Ps aeruginosa* have been demonstrated in 100 per cent of C F 's harbouring mucoid strains of *Ps aeruginosa* in the sputum whereas no precipitins against *Ps aeruginosa* could be demonstrated in sera from C F 's not harbouring *Ps aeruginosa* in the sputum (8, 15)

The purpose of this work was to study C F sera by means of Crossed Immunoelectrophoresis With Intermediate Gel (CIWIG) (26) in order to identify and quantitate the precipitins against *Ps aeruginosa* The reason for introducing this technique is that it is more sensitive and has a higher resolving power than the conventional gel precipitation techniques, *i e* it is possible to reveal a higher number of precipitins and, in the same operation to *identify* and *quantitate* the precipi

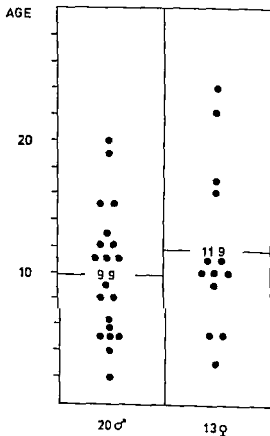


Fig 1 Sex and age distribution of the 33 C F pts The bars represent the mean ages of patients in the groups

electrophoresis modified by interposing a gel (the intermediate gel) between the first dimension gel and the St Ab-containing second dimension gel (reference gel) Serum from a pt is included in the intermediate gel During the second dimensional electrophoresis the antigens will migrate [illegible]

in the intermediate gel, the antigens (St Ag) will be a) completely-, b) partly–, or c) not retained by immunoprecipitation in the intermediate gel This is reflected in each of the corresponding precipitates of the *reference precipitate pattern* by a) absence of precipitate, b) fusion of reference and intermediate gel precipitate, the area of the former being reduced, and c) no change in the reference precipitate by comparing the testplate and a control plate without antibodies in the intermediate gel In this way the identity of the *antibodies of human serum can be determined using the standard system as reference* Free antigens in the serum under study will cause 'horizontal', straight lines of precipitates in the reference gel, perpendicular to the direction of the current The detailed theoretical basis of the interpretation of the results obtained by the CIWIG method is described in the publications of *Svendsen* & *Axelsen* (1972) and *Axelsen* & *Kirkpatrick* (in press)

The quantitation of the human antibodies were estimated by comparing the areas of the human precipitates *in the intermediate gels with the areas* of the corresponding precipitates of four standard plates in which St Ab were incorporated in the intermediate gels instead of human serum The titres of the human antibodies were expressed in arbitrary units in proportion to the four fixed concentration of St-Ab in the intermediate gels of the standard plates *Titre 5* $\geqq$ 20 µl/cm² > *titre 4* $\geqq$ 10 µl/cm > *titre 3* $\geqq$ 5 µl/cm² > *titre 2* $\geqq$ 1 µl/cm² > *titre 1* > 0 µl/cm²

Using the CIWIG method each serum was investigated for *the number of Ps aeruginosa* precipitins visible as immunoprecipitates in the intermediate gel, *the identity* (if possible) of the precipitins in relation to the reference system, and *the titres* of the identified precipitins in relation to the four fixed concentrations of St Ab

Chemicals

Trüche media 40 g peptone Orthana 2 g glucose, 5 g NaCl, 15 g Bacto Agar Difco 1 litre tap water, pH 7 5 *Coomassie brilliant blue*, Microme no 1137 was obtained from E Gurr Ltd London England *Agarose* for the electrophoreses was obtained from Litex Glostrup Denmark All the electrophoreses were run using the equipment of Dansk Laboratorieudstyr A/S Copenhagen Denmark

TABLE 1 *Distribution of Pseudomonas Precipitins on the Titre Classes and the Number of Patients with Precipitins of the Titre Classes*

Titre Class	No of *Precipitins*	No of C F Patients	
5	25	C F +MP	7
4	15	C F +MP	7
3	20	C F +MP	9
		C F +NMP	1
2	24	C F +MP	8
		C F +NMP	1
		C F (+)P	2
1	13	C F +MP	7
		C F (+)P	1
		C F —P	1
Non-Identified Precipitins	76	C F +MP	12
		C F +NMP	1
		C F (+)P	1
		C F —P	2

Occurrence of 173 *Ps aeruginosa* precipitins in serum from 33 C F pts. Ninety seven of the precipitins could be identified and quantitated and the distribution on the titre classes (for explanation see text) is given in the table The number (No) of pts with precipitins of each of the 5 *titre-classes* is also given together with the number of pts with non identified precipitins The C F pts are classified according to the bacteriological status of the sputum (for explanation of abbreviations see text to Table 2)

Freund's incomplete adjuvant was obtained from Difco Laboratories Detroit, U S A

RESULTS

Fig 2 A shows a control *CIWIG* in which 55 precipitates could be seen, Fig 2 B is a drawing of Fig 2 A When the St-Ag/St Ab ratio was altered 6 extra precipitates were recognized The precipitates were enumerated (not indicated at Fig 2 B) and provided the reference system for identification of the human antibodies in accordance with the principles discribed above Control experiments using Truche *media instead of St-Ag* in the first-dimension well did not reveal any precipitates

Precipitins against *Ps aeruginosa* were found in 20 out of the 33 sera (61 per cent)

1 CM

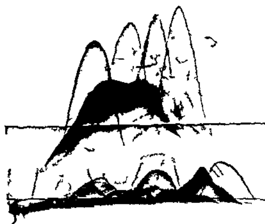


Fig 3 Crossed immunoelectrophoresis with patient s serum in the intermediate gel At least 17 different precipitates could be seen in the intermediate gel Identification and quantitation (for explanation see text) of 12 of these precipitins was possible (Electrophoresis and staining as in Fig 2A)

In these 20 sera a total of 173 precipitins against *Ps aeruginosa* were found Ninety-seven of the 173 precipitins (56 per cent) could be identified and quantitated, representing 30 different specificities The distribution of the precipitins on the titre classes and the number of pts with precipitins of the titre-classes are given in Table 1 It appears that the higest titres were seen in C F 's harbouring mucoid *Ps aeruginosa* in the sputum (C F s + MP) The positive sera contained from 1 to 22 precipitins each An example is shown in Fig 3 The distribution of precipitins on different groups of subjects is given in Table 2 from which it appears that the greatest number of precipitins was found in sera from C F 's + MP Sera from C F 's harbouring solely non mucoid *Ps aeruginosa* in the sputum (C F 's + NMP) and sera from C F 's who formerly harboured *Ps aeruginosa* but not at the time of this investigation (C F 's (+) P), were never found to contain more than 8 precipitins against *Ps aeruginosa* Sera from C F 's from whom *Ps aeruginosa* had never been isolated (C F 's – P), never contained more than 1 precipitin against *Ps aeruginosa*

Statistical analysis of the data recordered in Table 2 with regard to *precence/not presence* of *Ps aeruginosa* precipitins (Fourfold Table Test, Documenta Geigy) showed that *Ps aeruginosa* precipitins were found more frequently in sera from C F 's + P than in sera from C F 's – P ($p < 0\,01$) and C F 's (+) P ($p < 0\,05$) On the other hand, precipitins were not found more frequently in

TABLE 2 *Distribution of Pseudomonas Precipitins on Different Groups of Subjects*

Group of Subjects	No of Pts	No and Percentage of Pts with Serum Precipitins	Mean (x) and Range (r) of No of Precipitins per Pt with Serum Precipitins	
C F + P (Total)	17	15 (88%) (95% Conf Lim 98 5%–63 6%)	x 10 9	r 22 2
C F + MP	13	13 (100%) (95% Conf Lim 100%–75 3%)	x 11	r 22 2
C F + NMP	4	2 (50%)	x 5	r 8 2
C F (+)P	6	2 (33 1/3%)	x 4	r 7 1
C F —P	10	3 (30%)	x 1	r 1

Occurrence of *Ps aeruginosa* precipitins in serum from 33 C F pts classified into groups of subjects according to the bacteriological status of the sputum *+P* presence of *Ps aeruginosa*, *+ MP* presence of mucoid *Ps aeruginosa* *+NMP* presence of solely non mucoid *Ps aeruginosa* *(+)P* former presence of *Ps aeruginosa* but absence at the time of this study, *—P* absence of *Ps aeruginosa*

electrophoresis modified by interposing a gel (the intermediate gel) between the first dimension gel and the St Ab-containing second dimension gel (reference gel) Serum from a pt is included in the intermediate gel During the second dimensional electrophoresis the antigens will migrate through the intermediate gel and thereafter through the reference gel Depending on the concentrations of the corresponding antibodies of the pt s serum in the intermediate gel, the antigens (St Ag) will be a) completely b) partly , or c) not retained by immunoprecipitation in the intermediate gel This is reflected in each of the corresponding precipitates of the reference precipitate pattern by a) absence of precipitate b) fusion of reference and intermediate gel precipitate the area of the former being reduced and c) no change in the reference precipitate by comparing the testplate and a control plate without antibodies in the intermediate gel In this way the identity of the antibodies of human serum can be determined using the standard system as reference Free antigens in the serum under study will cause 'horizontal straight lines of precipitates in the reference gel perpendicular to the direction of the current The detailed theoretical basis of the interpretation of the results obtained by the CIWIG method is described in the publications of *Svendsen* & *Axelsen* (1972) and *Axelsen* & *Kirkpatrick* (in press)

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Precipitins against *Ps aeruginosa* were found in 20 out of the 33 sera (61 per cent)



Fig 5 Crossed immunoelectrophoresis with patient's serum in the intermediate gel Serum contained simultaneously 6 *Ps aeruginosa* antigens and at least 15 *Ps aeruginosa* precipitins Three of the antigens can be seen as faint lines of precipitates extending from one side of the reference gel to the other parallel to the border of the intermediate gel (horizontal) Reactions of identity are seen with 2 of the reference precipitates which are slightly elevated The other antigens caused very weak lines of precipitates parallel to the visible lines but closer to the anode However, they could not be made visible on the photography Four of the antigens and 9 of the precipitins could be identified and quantitated (for explanation see text) (Electrophoresis and staining as Fig 2A)

acute exacerbation He died 7 month later from his pulmonary disease

DISCUSSION

Prerequisites for the CIWIG method are a complex standard antigen (St-Ag) and a corresponding complex standard antiserum (St Ab) (26) The St Ag was produced by gentle extraction from 4 different strains of *Ps aeruginosa* in order to avoid loss of information due to denaturation and/or qualitative antigenic interstrain variation The St Ab was produced from a pool of sera from 10 rabbits in order to minimize the influence of the antibody response of the individual rabbit on the results The antibodies were purified and concentrated in order to minimize the background staining of the immunoplates and to detect as many precipitates as possible

A very high antigenic complexity of *Ps aeruginosa* is demonstrated, further investigation of the antigenic structure of *Ps aeruginosa* should be possible using the quantitative immunoelectrophoretic methods recently surveyed by *Axelsen & Bock* (1972)

The maximal number of *Ps aeruginosa* antibodies demonstrated in a single human serum has until now been 8 (27) By means of the CIWIG method we were able to demonstrate as much as 22 precipitins in a single serum This is probably due to the use of a more sensitive technique and not to differences in the series of patients

The finding of precipitins in all C F s + MP is in accordance with the findings by other authors (8, 15) In contrast to *Doggett et al* (1969, 1972) who did not demonstrate precipitins in the C F 's harbouring non mucoid *Ps aeruginosa* we found precipitins in 2 out of 4 pts in this group We also found antibodies in C F 's (+) P and in C F 's – P in accordance with *Habboushe et al* (1971) Differences in the sensitivities of the methods used by the different authors are probably responsible for the conflicting results as regards the last 3 groups of pts The statistical analyses showed significant differences between the antibody response in C F 's + MP and the other groups of pts, *i e* the C F 's + MP presented stronger and more differentiated antibody response

The percentage of identification of the human *Ps aeruginosa* precipitins obtained in this study (56 per cent) can probably be increased if the amount of human serum in the intermediate gels is lowered by which reactions of identity will be more numerous (26)

The identified precipitins were quantitated in 5 arbitrary titre classes and it appears from Tables 1 & 2 that maximal titres were reached by 25 precipitins (representing 13 different precipitin specificities) from 7 C F 's + MP In future studies it might be an ad-

vantage to subdivide titre 5 in order to discover changes of the strongest titres

It is interesting that 23 out of the 30 different identified precipitins only occurred in the group of C F 's + MP The significance of this finding will be subject to further study The remaining 7 different identified precipitins were distributed over all the groups In this connection it is noteworthy that the C F 's (+) P and the C F 's – P did not contain antibodies not found in other groups of subjects

The simultaneous occurrence of *Ps aeruginosa* antigens and precipitins against *Ps aeruginosa* in serum has not been described before It was not possible to unveil whether the pt had antibodies against these antigens but if so, the antigens must have been excess in the pt 's serum since they could be demonstrated as lines of precipitates in the reference gel

The interpretation of the strong and differentiated antibody response in the C F 's harbouring mucoid *Ps aeruginosa* in the respiratory tract is generally that mucoid strains are the actual pathogens in C F 's pts, on the assumption that infection raises antibodies whereas colonization does not raise detectable antibodies (8, 11 15) This is based on the clinical experience that pts whose sera contain antibodies against *Ps aeruginosa* are more severely affected than pts without antibodies (8) and that the clinical status of C F 's harbouring mucoid strains is worse than the clinical status of C F 's not harbouring mucoid *Ps aeruginosa* (8 11, 15), however, some authors (19) have not been able to find this relationship If mucoid strains were more pathogenic than non-mucoid strains the question arises, why these strains are rarely isolated from other pts infected with *Ps aeruginosa* Some kind of selection must be active in C F s since non-mucoid strains frequently precedes the mucoid strains (11, 13 14) The mechanism of this selection is obscure but factors correlated both with the C F host 14) and with the slime of the mucoid s ains 15 25) have been proposed to be re j nsible in this respect A C F specific factor is probably not working alone since *Burns & May* (1968) found that 10 per cent of pts suffering from bronchiectasis were harbouring mucoid strains of *Ps aeruginosa*, simultaneously presenting precipitins against *Ps aeruginosa* Pts from the same group, but harbouring only non mucoid strains, presented less frequently precipitins against *Ps aeruginosa* This situation is similar to the situation found in C F 's and thus, the interpretation of these findings might be that the selective factor favouring the mucoid strains is the immune response of the pts, i e owing to the great production of mucoid substance, the opsonizing effect of the antibodies on the bacterial cells of mucoid strains is inhibited as compared with the effect on bacterial cells of non mucoid strains

The demonstration of an *in vitro* antiphagocytic effect of mucoid slime (25) which is reverted by adding rabbit Pseudomonas antiserum to the test tubes is not adverse to our point of view, if the quantitative aspects of the mucoid slime produced be considered *Biggar et al* (1971) have postulated the possibilities of a quantitative or functional defect of IgA from C F 's specific for *Ps aeruginosa* which decreases the opsonic support to phagocytosis by alveolar macrophages According to our opinion the great amount of mucoid substance would emphasize the significance of such a defect

Generalized infections caused by *Pseudomonas aeruginosa* are rare occurrences in C F 's and thus, it cannot be precluded that antibodies play a role in that they keep the infection localized to the respiratory tract (5, 14) although the eradication of the infection is not successful

Doggett et al (1969, 1972) have postulated that high and increasing Pseudomonas antibody-titres can be a poor prognostic sign as 11 of their C F 's with high titres against *Ps aeruginosa* died because of respiratory insufficiency The mere finding of the very high number of precipitins as well as the high titres in the sera from C F 's + MP and the persistence of the infection in the respiratory tract could raise the question whether the local immune reactions in progress could enhance

the tissue damage of the respiratory tract of these pts thereby contributing to the poor prognosis of this disease

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Miss *Karen Falster* has skilfully performed the immunoelectrophoreses

The four *Ps aeruginosa* strains used have been 0 group typed by *O S Mikkelsen*, M D Statens Seruminstitut, Department of Clinical Microbiology, Århus Kommunehospital, Århus, Denmark

The sera were kindly obtained from *B Weeke*, M D, Protein Laboratory, University of Copenhagen, Denmark

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PASSIVE HEMAGGLUTINATION TEST, USING ERYTHROCYTES SENSITIZED WITH *KLEBSIELLA* TYPE SPECIFIC POLYSACCHARIDES

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A passive hemagglutination method has been used to determine antibodies against type specific capsular polysaccharide in the *Klebsiella* group Erythrocytes were coated with acidic capsular polysaccharide Chromium chloride was used as the coupling reagent The method has been tested on eleven serotypes

Determination of antibodies against the capsular, type specific polysaccharide antigens in the *Klebsiella* group, has usually been carried out by the precipitation method, probably due to lack of more sensitive methods *Landy et al* (6) reported years ago that lipopolysaccharide antigens of gram negative bacteria were able to sensitize erythrocytes, to be used in passive hemagglutination tests Other polysaccharides did not work in the same way, even treatment with alkali or acid before sensitization did not help

Recently a hemagglutination procedure for determination of antibodies against pneumococcal polysaccharide antigens, was introduced (1) Sheep erythrocytes were sensitized with purified type specific pneumococcal polysaccharides, using chromium chloride as the coupling reagent Provided that the pneumococcal polysaccharide was charged, it seemed to be suitable for erythrocyte sensitization by means of chromium chloride The sensitized erythrocytes were then used as antigen in the passive hemagglutination test

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All capsular, type specific polysaccharides in the *Klebsiella* group examined so far, contain uronic acid, as a charged group in the polysaccharide (7, 8) Conditions should therefore be present for sensitization of the erythrocytes with polysaccharides from this group by means of chromium chloride

The purpose of this paper is to see if antibodies against the capsular antigen in the *Klebsiella* group also could be determined by passive hemagglutination

MATERIALS AND METHODS

Antisera Rabbits were immunized as described earlier (3), with a broth culture of the different *Klebsiella* types The rabbits were bled from the ear, when test bleeding showed a high enough content of antibodies against the capsular polysaccharide to give a positive Ouchterlony gel precipitation read after 24 hr The precipitates were graded from + to + + + according to the quantity of the precipitate

Antigens The capsular polysaccharides from the different *Klebsiella* types were isolated as described earlier (2) Capsular polysaccharide antigens from, and antisera against the following types were produced

K. *pneumoniae* type 1, strain Sc
K. *pneumoniae* type 2, strain F 24
K. *pneumoniae* type 3, strain F 10, New York
K. *ozaenae* type 4, strain 4461
K. *ozaenae* type 6, strain F 101
K. *ozaenae* type AE, strain 366 and strain 047
K. type 7
K. type 11
K. type 14, strain 1193
K. type 21
K. type 35

Sensitization of erythrocytes The procedure was carried out as described in the paper about the pneumococcal polysaccharides, (1) only with a few adjustments for the *Klebsiella* polysaccharides

0 5 ml of washed and packed sheep (SRBC) or rabbit (RRBC) erythrocytes was mixed with 1 0 ml of saline, containing the *Klebsiella* polysaccharide 1 ml of 0 2 per cent chromium chloride ($CrCl_3$ 6 H_2O) in saline was added The chromium chloride solution was made just before use After thorough mixing, the suspension was left at room temp in the dark for five minutes, after which the erythrocytes were washed twice with 30–40 volumes of saline After washing, the erythrocytes were suspended to a 1 per cent suspension in saline, to be used as antigen in the passive hemagglutination test

When SRBC were used as carrier of the polysaccharide, the rabbit sera were absorbed with SRBC before dilution, to avoid hemagglutination by 'natural' antibodies The sera were inactivated by heating in a 56°C waterbath for ½ hr

The procedure was carried out in twofold dilutions of antiserum The saline, used for the dilution, was mixed with bovine serum albumin, to make a 0 2 per cent solution

The amount of antiserum used was 0 1 or 0 025 ml respectively and the same volumes of 1 per cent suspension of antigen coated erythrocytes were added After thoroughly mixing, the endpoint could easily be determined after two hours at room temp , but usually the titration trays were left over night at 4°C The tests were performed in duplicate or triplicate, and the results obtained were compared if both the macro- and the micro-method were used

The endpoint was expressed as the "reciprocal of the highest dilution of serum, giving distinct hemagglutination Normal rabbit serum was used as control

When 0 1 ml serum and 0 1 ml antigen suspension were used, the tests were set up in small tubes, or on hemagglutination trays (Manuplastic, Ltd London) The endpoint was read by tapping of the tubes without prior centrifugation, or by directly inspection of the hemagglutination tray This procedure is refered to as the macro-method

When 0 025 ml serum and 0 025 ml antigen were used, the equipment was Microtiter, produced by Cooke Engineering Comp Virginia, U S A The endpoint was determined by using the test reading mirror which provides magnification of the hem

by precipitation using the Biuret method (3) of determination of protein

RESULTS

The first examination was carried out in anti-*Klebsiella pneumoniae* type 3, and anti-*Klebsiella* type 11 sera, both against the homologous capsular polysaccharide antigen The procedure was performed with 0 1 ml serum Erythrocytes coated with heterologous polysaccharide, and without polysaccharide, were used as antigen control The results can be seen in Table 1

From the results obtained, it seemed that the passive hemagglutination test can be used for the determination of specific antibodies against the capsular antigen in the *Klebsiella* group too, at least for the two types tested

In the first examination, the polysaccharide concentration used was 1 mg/ml in saline

TABLE 1 *Hemagglutination of SRBC Sensitized with Type-Specific* Klebsiella *Polysaccharides*

Antiserum used	Polysaccharides, used to sensitize the SRBC		
	K pneum type 3 strain F 10 N Y	K type 11	None
K pneum type 3 strain F 10 N Y	4096*	4	2
K type 11	4	33 800*	2
Normal rabbit	4	4	4

* "Reciprocal" of serum dilution, giving distinct hemagglutination

TABLE 2 *Hemagglutination in Anti Klebsiella pneumoniae Type 3 Serum, Using SRBC Coated with Different Concentrations of Capsular Polysaccharide Antigen*

Conc of homologous polysaccharide used for coating SRBC	* Reciprocal of serum dilution, giving distinct hemagglutionation	Comments to determination of endpoint
2 mg/ml	2560	Difficult to see endpoint
1 5 „	3840	Endpoint, easy to see
1 0 „	3840	„ „
0 5 „	3840	„ „
0 25 „	–	Difficult to read the endpoint, because of hemolysis
0 10 „	–	
0 0 „	–	

TABLE 3 *Hemagglutination Titre and Quantitative Antibody Determination in Anti Klebsiella Sera from Rabbit No 7 and No 9, both Immunized with Klebsiella Type 11*

Serum no	*Hemagglutination titre**		Specific antibody mg/ml serum
	macro	micro	
107 (7)	12 000	24 000	
117 „	12 000	24 000	1 53
136 „	96 000	192 000	1 91
137	48 000	24 000	1 86
145 „	12 000	12 000	1 49
108 (9)	12 000	12 000	1 49
138 „	12 000	12 000	1 17
147	24 000	24 000	1 81
153	24 000	48 000	1 68
168 „	48 000	96 000	1 68
172	24 000	12 000	1 43

* *Reciprocal of serum dilutions giving distinct* hemagglutination

The procedure was repeated for anti *Klebsiella pneumoniae* type 3 serum but now with different concentrations of polysaccharide The results are presented in Table 2

Concentrations of 1 5, 1 0 and 0 5 mg polysaccharide per ml saline seemed to have the same coating effect on the erythrocytes By using 0 25 mg/ml or less, the SRBC were hemolyzed by chromium chloride The poly saccharide seemed to protect the erythrocytes against the chromium chloride

For the following examinations the concentration of polysaccharide for coating is 1 mg/ml

From Table 1, a weak unspecific reaction between rabbit sera and SRBC can be observed By using rabbit erythrocytes (RRBC) this reaction should be excluded Hemagglutination was carried out in six rabbit anti *Klebsiella* type 11 sera using RRBC coated with polysaccharides

The results obtained by using RRBC instead of SRBC seemed to vary very little The following titrations have been carried out with SRBC The reason for doing this is that SRBC seem to keep longer in the refrigerator compared with RRBC which are more easily hemolyzed by storing To avoid the weak unspecific reaction between rabbit sera and SRBC mentioned above, all rabbit sera are absorbed with SRBC before dilution

For two rabbits both immunized with *Klebsiella* type 11 over a long period of time, specific antibodies against the capsular polysaccharide were determined by this passive hemagglutination method both in macro- and in microscale The examinations were not performed on the same day, nor with the same antigen preparation.

Since there is no suitable way to determine the amount of polysaccharide fixed to the erythrocytes for each antigen preparation, the examinations in microscale were repeated, this time with the same antigen Quantitative determination of specific antibodies against capsular polysaccharide was carried out at the same time in the same sera

From the results reported in Table 3 it seems that the reproducibility of the method

TABLE 4 *Hemagglutination in Rabbit Antisera from Different Klebsiella types against their Homologous Capsular Polysaccharide Antigen*

Absorbed rabbit antisera against	Gelprec	Hemagglutination titre*
K pneumoniae		480
type 1, strain Sc	+ +	384
K pneumoniae	+ +	2 400
type 2, strain F 24	+	2 400
K pneumoniae	+	480
type 3, strain F 10	+ + + (1 54)	19 200
K ozaenae, type 4		
strain 4461	+	240
K ozaenae, type 6	+ + + (0 58)	2 400
strain F 101	+ +	1 200
K ozaenae, type AE	+	240
strain 366	+	240
K ozaenae, type AE	+	600
strain 047	+	96
K type 7	+	4
" "	+	4
" "	+ + + (053)	3 840
" "	+ +	4
K type 11	+ + +	19 200
" "	+ + +	12 000
K type 14	+ + +	7 680
strain 1193	+ + +	7 680
K type 21	+	1 920
" "	+ + + (0 96)	19 200
K type 35	+	480
" "	+ (0 10)	600

* 'Reciprocal' of serum dilution giving distinct hemagglutination The numbers in parenthesis report to mg antibodies precipitated by homologous polysaccharide in one ml serum

is good The difference in the results from the macro- to the microtechnique, is not above one dilution step The same difference can occur when the micromethod is repeated with another antigen preparation

By comparing results obtained by passive hemagglutination and quantitative precipitation, the table shows that hemagglutination titres in the range of 12 000 to 192 000 correspond to 1 2 to 1 9 mg antibody/ml serum at least for anti *Klebsiella* type 11 sera

Since all the polysaccharides in the *Klebsiella* group examined so far contain uronic acid as the charged monosaccharide, most likely, the hemagglutination method can be used for determination of type specific antibodies in all type specific antisera

Passive hemagglutinations were therefore carried out in 27 rabbit antisera belonging to 11 different *Klebsiella* types The homologous polysaccharides were coated on SRBC and used as antigens Normal rabbit sera were used as serum control, and SRBC without polysaccharide, as antigen control All sera showed a positive Ouchterlony gel precipitation test, arbitrarily graded from + to + + + according to the quantity of the precipitate For five of the sera, a quantitative precipitation was also carried out The results are shown in Table 4

DISCUSSION

Landy & coworkers many years ago reported that lipopolysaccharide antigens of gram-negative bacteria were able to sensitize erythrocytes for use in hemagglutination tests The sensitization was working without any

coupling agent On the other hand, various chemicals have also been used to bind antigens to erythrocytes for hemagglutination examinations (6) Bis diazotized benzidine and tannic acid are probably best known The procedures have, however, often the disadvantage to be complicated and timeconsuming

In contrast, when chromium chloride was used as coupling reagent, to bind protein-antigens to the red cells, the procedure was quick and easy to perform (4) To use chromium chloride in serological examinations with non protein antigens was unsuccessful, until the work using pneumococcus polysaccharide antigen was reported (1) Erythrocytes, sensitized with charged pneumococcal polysaccharides were used as antigens, not only in passive hemagglutination test, but also for hemolysis in gel detection of specific antibody and antibody producing cells

The procedure reported in this paper, has been used for determination of specific antibodies against the charged capsular polysac-

showed that they also could be used for sensitization of erythrocytes, using chromium chloride as coupling agent Chromium chloride has a destructive effect on the erythrocytes but when mixed with a solution of charged polysaccharide and chromium chloride at the same time, the erythrocytes were stable

In this method 1 mg polysaccharide per ml saline was used for coating Smaller amounts were not enough to protect against hemolysis, and by using bigger amounts, it was difficult to read the end point

A drawback of the method is the lack of ways to determine the exact amount of polysaccharides fixed to the erythrocytes But by using well standardized conditions, it seems as if the erythrocytes are coated to the same extent, showed by the reproducibility of the method The variation of the results is usually not more than one dilution in either direction if any at all

Because of the different structure of the capsular polysaccharides in the *Klebsiella* group, great care should be taken, to avoid comparison between the different types The erythrocytes can perhaps be sensitized to different extent all dependent of the specific determinants in the polysaccharide

The procedure is easy and quick to carry out and the reproducibility is good When only small quantities of antisera are available, the hemagglutination method can be of great help, specially by using the micro procedure

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AGGLUTINATION AND IMMUNOFLUORESCENCE STUDIES ON SURFACE ANTIGENS OF *S. AUREUS*

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Serological typing of 200 *S aureus* strains from clinical specimens has been performed with the Oeding Haukenes factor sera Since weak agglutinations were recorded serologically reacting antigens were studied in immunofluorescence with FITC labelled type specific anti *S aureus* $F(ab')_2$ fragments of IgG and IgM and TRITC labelled normal IgG to detect protein A by its reaction with the Fc part of IgG Protein A was found to be unevenly distributed among the *S aureus* cells derived from a single colony and, by trypsin digestion of the bacteria type specific antigens were found to be masked by the common cell surface constituent, protein A

Three major cell wall components of *Staphyl ococcus aureus* have been identified and extensively characterized the backbone mucopeptide to which ribitol techoic acids are bound as well as protein A (28, 29) Several reports have indicated that protein A is situated in the outermost part of the staphylococcal cell wall (17, 18, 30), and evidence has been presented that protein A is unevenly distributed on bacteria derived from a single colony (17) Attempts to distinguish other antigens using agglutinin-adsorption have been partially successful (6, 15, 19, 21, 23), the capacity of protein A to react with the Fc-part of IgG (8) must have influenced the results however since incomplete removal of IgG from a serum absorbed to give a specific factor serum results in reactions due to the interaction of protein A and the Fc part of IgG (11)

In a previous report from our laboratory, the use of IgM or F(ab) fragments of IgG in attempts to further characterize the antigens on the cell surface of *S aureus* was suggested (11) In the work described here, serotyping of *S aureus* with factor sera based on IgM has been performed and the antigens on the cell surface of *S aureus* further studied using FITC labelled anti *S aureus* F(ab') fragments of IgG or IgM

The results indicate that protein A is an important antigen masking type specific antigens and that trypsin treatment of the bacteria can be used to uncover type antigens

MATERIALS AND METHODS

Strains and Culture Technique

The Oeding type collection of *S aureus* strains *S aureus* Cowan I, II, and III, and *S aureus* [illegible]

agar Experiments were done using 5 and 18 [illegible] cultures on nutrient agar and 18 hr cultures on Mannitol salt agar

Sera and Adsorption Technique

Pooled serum from 25 nonimmunized rabbits was used as normal serum Antistaphylococcus serum

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was obtained by imunizing rabbits according to *Oeding* (21) Factor sera a_4 a_5, b_1, c_1, e h_1 h_2 k_1, k_1k_2, $\imath_1\imath$ m n 263-1, 263 2 were produced from

sis (21) Agglutinin titres were 1/10–1/40 If re adsorbed with the agglutinating strains the factor sera were shown to be mono- or bispecific as indicated by *Oeding & Haukenes* (15 21) $F(ab')_2$ fragments were adsorbed using the lowest dose of bacteria giving an adsorbed serum negative in agglutination or immunofluorescence for the strain used for adsorbtion Adsorbtion was carried out for 2 hr at 37°C and then for 18 hr at 4°C Agglutination was done as described earlier (11)

Immunofluorescence Procedure

IgG and $F(ab')_2$ fragments of IgG were prepared from antistaphylococcus serum and conjugated as described earlier (9, 12) using a dye to-protein ratio of 1/50 The conjugated fragments (6 6 mg/ml) had F/P ratios (µg/mg) 7–9, and a single unbroken line was seen in immunoelectrophoresis against donkey anti rabbit plasma protein serum (27) Normal rabbit IgG was conjugated with tetramethylrodamine iso-thiocyanate (TRITC) as described by *Goldman* (13)

Staining of smears and recording of reactions were done as described previously (9) In experiments with adsorbed anti *S aureus* $F(ab')_2$ fragments the number of fluorescent bacteria per total number of bacteria was calculated by counting the same area of the slide in visual light microscopy as well as in UV microscopy

Trypsin Treatment

Heat fixed bacterial smears were treated with trypsin for 10 20, 30, 40, 60, and 120 min at pH 7 4 and 37°C using a 0 3 per cent trypsin solution in PBS (18)

Protein A content was determined as described earlier (7)

RESULTS

Serological Typing of S aureus *According to Oeding Haukenes*

200 S aureus strains isolated from clinical specimens were serotyped with factor sera a_4, a_5 b_1, c_1, e, h_1, h , k_1, k_1k_2, $\imath_1\imath_7$, m n 263 1, and 263 2 10 strains (5 per cent) agglutinated spontaneously The most frequent antigenic patterns were k_1k_2, h_2 47 5 per cent, h. k_1k_2, 263 2 21 5 per cent, a_5, h_2, k_1k_2, 263 2 8 7 per cent, and c_1, $\imath_1\imath_7$ 7 5 per cent Three strains were not typable with the factor sera used In all, 45 different patterns were recorded, and no correlation to clinical source was found The titres of the factor sera were

TABLE 1 *Immunofluorescence Reactions of Adsorbed and Nonadsorbed FITC labelled Anti* S aureus *1503 F(ab')₂ Fragments with* S aureus *Strains grown on Nutrient Agar or Mannitol Salt Ager*

S aureus strains (protein A production[a])	Nonadsorbed $F(ab')_2$ fragments Test strain grown on nutrient agar	$F(ab')_2$ fragments adsorbed with *S aureus* Cowan I Test strain grown on	
		nutrient agar	Mannitol salt agar
1503 (1 500–3 000)	3+	3 +/–[d]	2+
2253 (1 500–3 000)	3+	3 +/–[d]	2+
9 Oeding strains[b] (375 3 000)	2–3+		–
4 Oeding strains[c] (< 375)	–		–

[a] nanogram/10⁹ bacteria

[b] strains CI CIII, 365 3647 F21, 17A 3189 2095 670

[c] strains CII W46, 5687, 263

[d] approximately 1 2 bacteria with 3+ fluorescence per 100 bacteria Unevenly distributed fluorescence and varying degrees of intensity of some bacteria while others were not stained Trypsin digestion of bacteria showed an approximately tenfold increase in the number of cells with 3+ fluorescence



b

c

a) Nonadsorbed $F(ab')_2$-fragments, test strain not trypsin digested
b) Adsorbed $F(ab')_2$-fragments, test strain not trypsin digested
c) Adsorbed $F(ab')_2$ fragments, test strain trypsin digested for 20 min

low 1/10–1/40 and the agglutinations weak Results obtained by two independent investigators were not always consistant

Group- and Type-Specific S aureus *Antigens in Immunofluorescence*

To identify group and type antigens of Cowan and Oeding type strains FITC-labelled anti-*S aureus* F ab fragments of IgG were used in order to include only specific antigen-antibody reactions and not reactions between the Fc-part of IgG and protein A

Using $F(ab')_2$-fragments from antiserum to *S aureus* strain 1503, producing a high amount of protein A, a smooth, uniform, strong to moderately strong fluorescence was seen at the periphery of all strains producing high to moderate amounts of protein A (> 375 nanog/10^9 bacteria) (Table 1) Anti-*S aureus* 1503 $F(ab')_2$-fragments were adsorbed with Cowan and Oeding type strains to obtain type-specific fragments $F(ab')_2$-fragments adsorbed with strains with high protein A production stained only the homologous strain (1503) and a strain (2253) reported to have similar type-antigens (14). A

marked heterogeneity in staining of individual bacteria even from a single colony by the adsorbed $F(ab')_2$ fragments was noted Some cells showed brilliant 3+ staining some showed irregular fluorescent margins with varying degrees of fluorescence, and most were completely unstained

To study further the relation between type specific antigens and protein A double tracing was performed with the adsorbed FITC labelled anti *S aureus* 1503 F(ab') fragments to identify type antigens and with TRITC labelled normal rabbit IgG to identify protein A by its reaction with the Fc part of IgG The number of cells with a green 3+ reaction due to a reaction with type specific antigen was the same as that detected if only type specific FITC labelled F(ab') fragments were used while the rest of the cells showed orange red fluorescence due to protein A

S aureus 1503 was treated with trypsin (18) for various lengths of time and stained with FITC labelled anti 1503 $F(ab')_2$ fragments adsorbed with a strain with high protein A production and TRITC labelled normal rabbit IgG (Fig 1) The orange red fluorescence due to protein A disappeared after approximately 10 min of trypsin treatment If the bacteria were treated for approximately 20 min the number of cells showing green fluorescence increased to about 10 times the number on a nontreated slide Longer trypsin digestion weakened the fluorescence reaction and after 2 hr no fluorescent cells could be seen indicating that the type specific antigens are trypsin sensitive The result shows that there was increased exposure of type specific antigens in trypsin treated cells whereas protein A as has been shown earlier (18) was degraded

Cultivation of *S aureus* on Mannitol salt agar has been shown to suppress the production of protein A (18) and was therefore tried in attempts to eliminate a surface layer of protein A Nonadsorbed and adsorbed anti *S aureus* 1503 F(ab') fragments reacted with a uniform 2+ staining of all cells with the homologous strain (1503) and with the antigenically similar strain 2253 after cultivation on Mannitol salt agar The experiments with bacteria grown on Mannitol salt agar show that the surface layer of protein A is eliminated thus exposing type antigens, the type specific reactions obtained were weakened however, indicating that the type specific antigen had also been affected by growth on Mannitol salt agar

Results similar to those reported for strain 1503 were observed with FITC labelled anti *S aureus* 3647 and 5687 $F(ab')_2$ fragments or IgM while strains 17A F21 and 365 could not be studied possibly due to a low level of type specific antibodies in the sera used

DISCUSSION

Cowan (6) showed that *S aureus* strains can be divided into three clear-cut groups and one atypical group by slide agglutination Several later studies have confirmed *Cowan's* findings and further subdivisions especially of the atypical group have been attempted (4, 15 16 19 21 23) *Christie & Keogh* (4) *Hobbs* (16) *Pillet* (23) and *Pillet & Orta* (24) tried to find by agglutinin adsorption sera reacting only with the homologous strain within a set of type strains titres were usually low however or homologous as well as heterologous antibodies were removed Troublesome crossreactions were never eliminated A system by which antigens are identified by a pattern of agglutination with a set of type specific factor sera has been worked out by *Oeding* (19 21) The system was further elaborated by *Haukenes* (15) through crossadsorption of the original Oeding factor sera The main groups of the Oeding system correspond to the three Cowan groups (20) Apart from the fact that there is general agreement as to the validity of the three Cowan groups studies to reveal type specific antigens of *S aureus* have been only partially successful (15 16 19 21 22 24) The presence of a strong common antigen (16 25) and the similarities of the type antigens (16 25) have been discussed as possible explana

tions of the number of crossreactions reported *Pillet et al* (25) found that papain digestion of *S aureus* strains almost completely eliminated their adsorbing capacity and that the papainsensitive substance could be extracted from the cells by heat treatment They concluded that a major common antigen exists Attempts to eliminate common antigens in favour of type specific antigens useful in serotyping work have included growth on different media, different times and temperatures of incubation heat treatment and treatment by proteolytic enzymes (2 3 15 19, 20, 26)

In a previous report, *Forsgren & Forsum* (11) showed that IgG from rabbits immunized with *S aureus* strains agglutinates the strains used for immunization and antigenically related strains and, in addition strains containing a significant amount of protein A due to a reaction between protein A and the Fc part of IgG The $F(ab')_2$ fragments obtained by enzymatic digestion with pepsin of the IgG molecules give a specific agglutination reaction with *S aureus* strains containing antigens in common with the strains used for immunization Using highly specific FITC labelled anti enterotoxin B IgG and its $F(ab')_2$ fragment *Forsgen et al* (10) showed that intact IgG is not suitable for the detection of antigens other than protein A on the cell surface of *S aureus* in immunofluorescence For such purposes the use of F(ab) fragments was recommended *Forsgen & Forsum* (11) further showed that careful adsorption with *S aureus* strains to produce specific factor sera will remove IgG but that the adsorbed serum still agglutinates the strains used for immunization and strains with antigens in common with this strain IgM was shown to be responsible for this agglutination

In the present study the agglutination studies have been extended to cover the complete Oeding Haukenes scheme It is concluded that

1) all factor sera prepared as recommended by *Haukenes* contain IgM and no IgG as shown by immunoelectrophoresis
2) the factor sera are mono- or dispecific as indicated by *Haukenes* in the system used if all IgG is removed
3) it is possible to type virtually all *S aureus* strains using the Oeding Haukenes factor sera The antigenic patterns found agreed with those found by *Akatov & Marandon* (1) in strains isolated in the USSR
4) the system has the disadvantage of low agglutinin titres 1/10–1/40 making reading of the slides difficult and subject to some degree of individual variation

Virtually all *S aureus* strains investigated have been shown to produce protein A (7) Studies of strains producing high amounts of protein A have shown that protein A is a trypsin sensitive surface component of *S aureus* which is evenly spread over the bacterial surface (18 30) In a study of the distribution of protein A amongst strains of *S aureus* *James & Brewer* (17) found several strains to be electrophoretically heterogeneous, the heterogeneity was abolished after trypsin treatment It was suggested that the original heterogeneity was due to the presence of different amounts of protein A on the surface of different cells and that removal of the protein A by trypsin treatment leaves a surface that consists essentially of the glycopeptide to which techoic acid still is attached Using adsorbed and nonadsorbed FITC labelled F(ab) fragments of anti *S aureus* IgG and FITC labelled IgM it has been shown in this study that protein A is one group specific antigen on the cell surface of *S aureus* Adsorption with a strain producing high amounts of protein A produced fragments with a high degree of type specificity however the type antigens were unevenly expressed some of the bacteria not being stained *Cohen & Oeding* (5) reported an apparently similar phenomenon but no explanation was offered Double tracing showed protein A to be the surface constituent not stained by the type specific fragments By light trypsin treatment an increasing portion of type specifically staining bacteria was recorded, suggesting that protein A

masked other antigens in most of the bacteria Since protein A is defined by its reactivity with the Fc-part of IgG and since an increasing number of type specifically fluorescent cells could be seen after light trypsin treatment while longer trypsin treatment also destroyed type antigens, it might be that type-specific antigenic sites on protein molecules with protein A reactivity are not fully expressed in the intact molecule The light trypsin treatment thus exposed these sites

The finding that protein A masks other antigenic sites on *S aureus* strains provides a reasonable explanation of the low agglutinin titres found in type-specific factor sera It appears that bacteria which lack protein A would be of advantage in serotyping work since protein A is group specific and masks other antigenic sites Culture of *S aureus* on Mannitol salt is known to suppress protein A production by *S aureus*, the findings in this report suggest, however, that also type-specific antigenic sites are affected Trypsin treatment for 20 min destroys protein A while other antigenic sites serologically are left reactive Trypsin digestion could therefore be of value in serotyping of *S aureus*

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IMMUNODIFFUSION STUDIES OF PNEUMOCOCCAL ANTIGENS WITH SPECIAL REFERENCE TO SEROTYPE 27

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The antigenic pattern of a pneumococcal strain of serotype 27 used as model was studied by immunodiffusion methods and found to show a complex antigenic structure with a least 21 separate precipitinogens Strains belonging to type 11 28 and 29 contained most of the precipitinogens present in the model strain while the antigenic composition of type 3 strain differed more from this strain Double diffusion studies of capsular polysaccharides prepared by conventional methods revealed the presence of contaminating C polysaccharide Within the tested pH range 4 9 the capsular polysaccharides migrated toward the anode The electrophoretic migration of the C polysaccharide varied with pH At pH 4 5 it moved slowly toward the cathode which made it possible by preparative electrophoresis, to obtain capsular polysaccharide free from the contaminating C polysaccharide This procedure is suggested as a final step in isolation of capsular polysaccharides for serological work

The classical work of *Heidelberger & Kendall* (10) provided much of the present knowledge of the immunochemistry of pneumococci This work was largely focused on the capsular polysaccharides, the specific soluble substances (SSS) Practically no work has been published in which the antigenic mosaic of *Diplococcus pneumoniae* has been studied by means of methods with high resolving capacity such as double diffusion in gel and immunoelectrophoresis When applied for studies of other bacteria these methods have revealed an antigenic complexity not known from analyses by other methods (5 12 14) The purpose of the present investigation was to study by methods of immunodiffusion techniques the antigenic composition of various preparations of pneumococci including the hitherto immunologically uncharacterized nucleoprotein (15) and M-protein (1) preparations For the future study of the immune response to SSS in patients with pneumococcal infections a further aim of this investigation was to find a method by which to separate conventionally purified SSS from contaminating C-polysaccharide (9)

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MATERIAL AND METHODS

Bacterial strain As a model strain was used a smooth strain of serotype 27 designated 27 D In addition, smooth strains of the serotypes 2, 3 11, 28 and 29 were used as well as another smooth strain of serotype 27 The latter strain (27 RS) was derived from a rough variant 27 R, by repeated mouse passages This rough variant strain as well as a rough variant of serotype 2 were also employed in the investigation They had both a typical rough colony appearance showed no observable capsular swelling reaction and were apathogenic if injected intraperitoneally into white mice

Antigens Antigen for immunization was prepared

from the model 27 D strain The bacteria were cultivated in fluid antigen free medium (13) at pH 6 9 for 8 hr, and the bacterial suspension was freeze pressed according to *Edebo* (6)

Seven types of antigen preparations were used for immunodiffusion analyses

FPA Freeze pressed antigens (FPA) were prepared from each of the strains and referred to as FPA, FPA_3 FPA_{11} etc where indices refer to serotype The bacterial strain in question was cultivated in meat extract broth (4) to which 3 per cent glucose, 0 03 per cent cystein hydrochloride and 0 5 per cent yeast extract (Difco 0127 01) was added at 37° C for about 8 hr with continuous adjustment of pH to 6 9 with 5N NaOH After centrifugation at 1500 × g for 30 min at 4° C the bacterial sediment was washed three times in sterile buffered 0 9 per cent NaCl (0 075 M sodium phosphate pH 7 0) and resuspended in 1/10 of the original volume of this buffer and homogenized in a Serwall omnimixer The suspended bacteria were disintegrated by freeze pressing (6) whereupon the material was shaken at 4° C for 16 hr After centrifugation at 27,000 × g for 30 min the supernatant was collected and used as antigen From dry weight measurements the concentrations of these FPA antigens were calculated to 0 8 4 6 per cent The protein content was estimated by the Lowry method (17) using tyrosine as standard and the values ranged between 1 18 mg per ml

RDA On aliquot of the bacterial suspension described above was disintegrated from the model strain in a Ribi cell disintegrator (19) This antigen was designated RDA_{27D}

M protein The procedure described by *Austrian & McLeod* (1) was followed for preparation of M protein from the model strain

Nucleo protein Antigen from the model strain was prepared according to *Heidelberger & Kendall* (10) as modified by *Jyssum* (15)

Crude polysaccharide Antigen was prepared as follows The supernate after centrifugation of bacteria grown in antigen free medium (12) at 37° C for 3 days was concentrated 10 times by evaporation and dialysed against buffered saline

Purified SSS ?7 was prepared from the model strain according to the so-called third procedure of *Jyssum* (15)

Purified C polysaccharide was prepared from the rough type 2 strain according to the third procedure of *Jyssum* (15

Antisera The freeze-pressed antigen from the model strain prepared as described above was used for immunization in mixture with Freund s complete adjuvant Three goats were hyperimmunized by means of weekly subcutane s injections of slightly increasing amounts of a gen (2 7 ml) Based upon test bleedings 150 ml of blood was taken from the best responding a nal The serum designated anti 27D was dispe ed in aliquots which were kept at -30° C until use No preservations were added Anti C polysaccharide sera were prepared in rabbits according to *Goebel & Adams* (7)

Rabbit antisera against specific soluble substances from all of the employed serotypes were the commercial ones from Statens Serum nstitut Copenhagen which are generally used for capsular swelling (18) These sera are designated anti SSS 3 anti SSS 11 etc

Immunodiffusion techniques The double diffusion in gel method of Ouchterlony was employed in the gel chamber modifications described by *Wadsworth* (21) and *Holm* (11) Immunoelectrophoresis was performed as described by *Wadsworth & Hanson* (22)

Block electrophoresis Horizontal block electrophoresis was performed using Sephadex G 25 (Pharmacia, Uppsala Sweden) as supporting medium principally as described by *Holmgren et al* (14) A slurry of the Sephadex was made with an acetate buffer (pH 5 0) and spread 2 mm thick on a 20 × 10 cm glass plate with long side plexi glass frames The antigen was applied in a transverse basin cut in the gel The electrophoretic run was performed at 3 V/cm for 12 hr whereupon the block was cut transversely in serial 3 cm strips which were then eluted with distilled water and the fractions obtained were tested using the double diffusion in gel method

RESULTS

Studies of antigens of the model strain The various antigenic preparations from the model strain (27 D) were studied in double diffusion and immunoelectrophoretic analyses with antiserum against this strain (anti 27 D) FPA_{27D} and RDA_{27D} both resulted in precipitation patterns consisting of at least 21 separate immunoprecipitates In comparative immunodiffuison analyses the precipitinogens of the two preparations were shown to be serologically identical After heating to 56° C for 30 min several of these precipitinogens were no longer demonstrable and after heating to 80° and 100° C for 15 min 12 and 9 precipitinogens only were shown Six precipitinogens were still demonstrable in RDA_{27D} after autoclaving at 120° C for 30 min

The M protein preparation from the model strain gave a much less complex pattern in immunodiffusion analyses with anti 27D consisting of only four lines In comparative

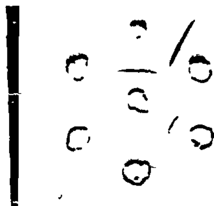


Fig 1 Double diffus on analysis of the SSS 27 preparation (central well) and clockwise in the peripheral basins start ng with the upper well anti SSS 27, crude polysaccharide antigen anti C polysaccharide C polysaccharide preparation saline and C polysaccharide preparation

double diffusion analyses these lines all fused with precipitates formed by RDA_{27D} and anti 27D The nucleoprotein preparation contained most of the precipitinogens demonstrable in the RDA_{27D} After absorption with the nucleoprotein preparation the anti 27D antiserum contained precipitins to 6 of the precipitinogens in the RDA_{27D}

Crude polysaccharide from the model strain as well as purified SSS 27 resulted in one dense precipitate with anti SSS 27 (Fig 1) Also in immunoelectrophoretic analyses only one precipitate was formed between SSS 27 and anti SSS 27 The same pattern was obtained with autoclaved SSS 27 The localization of this precipitate indicates an electrophoretic mobility of SSS 27 similar to that of human serum β_2 globulin No precipitate was formed between SSS 27 and anti 27D

The C polysaccharide preparation, unheated or autoclaved, gave, one precipitate in double diffusion analyses if anti C polysaccharide serum were used (Fig 1) Anti C polysaccharide serum also revealed the presence of C polysaccharide in the crude polysaccharide preparation from the model strain as well as in the purified SSS 27 (Fig 1) Immunoelectrophoretic analyses revealed that the electrophoretic mobility of the C polysaccharide was pH dependent At pH 4 0 and 5 0 a migration toward the cathode occurred (Fig 2) while the mobility was negligible at higher pH This circumstance made it possible in preparative block electrophoresis at pH 5 0 to obtain capsular polysaccharide free from contaminating C polysaccharide as tested by the double diffusion method This is illustrated in Table 1 showing the results obtained with SSS 27

Differences in antigenic composition at the



Fig 2 Immunoelectrophoretic analys s (pH 5 0) of C polysaccharide preparation (circular well) and anti C polysaccharide (long tudinal bas ns) Anode to the right

TABLE 1 *Double Diffusion Analyses of Eluate from Block Electrophoretic Fractions of SSS 27 Run at pH 5 0*

Precipitation with	Electrophoretic fractions*											
	—6	—5	—4	—3	—2	—1	+1	+2	+3	+4	+5	+6
anti SSS 27	—	—	—	—	—	+++	+++	++	+	—	—	—
anti C	—	—	—	—	—	++	—	—	—	—	—	—

* eluted from 3 cm sections of the block + indicates anodal migration — cathodal

21*

TABLE 2 *Number of Precipitates Formed Between* RDA_{27} *and Aliquots of Anti 27D Absorbed with FPA from Different Types of Pneumococci*

Anti 27D absorbed with	No of precipitates with RDA_{27}	
	non autoclaved	autoclaved
FPA	5	0
FPA_3	12	0
FPA_{11}	6	0
FPA_7	0	0
FPA_{28}	7	0
FPA_9	5	0

model strain and other strains To identify precipitinogens in the model strain not present in bacteria of other serotypes and to investigate their thermoresistance, anti 27D was absorbed with FPA_3 FPA_{11}, FPA_7, FPA_8 or FPA_9 whereupon the absorbed antiserum aliquots were tested by the comparative double diffusion method against untreated and autoclaved RDA_{27D} The results of these experiments are shown in Table 2 From further comparative double diffusion analyses employing these absorbed antiserum aliquots it could be concluded that 3 of the precipitinogens of the model strain were not present in any of the other serotypes studied C polysaccharide, SSS 27 and M protein preparation factors did not constitute any of these precipitinogens

Comparison of a rough strain of type 27 (27R) and its corresponding smooth strain (27RS) FPA $_{27RS}$ contained SSS 27 which resulted in a dense precipitate with anti SSS 27 The FPA_{27R} freeze dried and re dissolved in a minimal volume of distilled water to obtain a maximal concentration caused a deviation at comparative immunodiffusion analyses of the precipitation line between SSS 27 and anti SSS 27 showing that also the rough variant contained traces of SSS 27 Three precipitinogens present in FPA_{27R} could not be demonstrated in FPA_{27RS} but absorption of anti 27D with FPA_{27R} eliminated all precipitins against FPA_{27RS}

Electrophoretic mobility of SSS from different serotypes Crude polysaccharide antigens from serotypes 3 11 28 and 29 were tested in immunoelectrophoresis with their corresponding anti SSS antisera The anodic mobility of these polysaccharides differed SSS 3 being the most rapidly migrating substance If the pH in the electrophoretic runs was varied within the range 4 0 9 0 lowered pH resulted in an increased migration rate of the capsular polysaccharides This mobility change, however, was not pronounced except in the case of SSS 3 where it was marked

DISCUSSION

By double diffusion in gel and immunoelectrophoretic methods the antigenic mosaic of the pneumococcus was shown to be very complex as has also been observed in cases of other bacteria studied by such methods *e g* *Streptococcus* (12) *Escherichia* (14) and *Neisseria* (5) At least 21 precipitinogens were revealed in a type 27 pneumococcus strain used as model This shows that the concept of the antigenic composition of pneumococci bacteria based upon capsular polysaccharide, C polysaccharide Forssman antigen nucleoprotein and M protein is far from complete

The capsular polysaccharides and the C-polysaccharide were not represented in the multilinear reference system formed by RDA_{27D} and its homologous antiserum This is in accord with the early observation of *Avery & Neill* (2) who noted that disintegrated pneumococci on immunization give rise to an antibody response to the nucleoprotein fraction but not to the polysaccharides. In contrast, immunization with intact bacteria results in antibody formation also to the polysaccharides especially to the capsular one

The capsular polysaccharide was the first known antigen that enabled separation of pneumococcal strains into different serological types Some early observations (2) had however, indicated the occurrence of antigenic differences between pneumococcal strains unrelated to the capsular antigen The present study shows that the investigated pneumococcal strains have many antigens in

common but that differences exist which are not related to the capsular antigen It is possible that extended studies of such antigenic differences may be of help for a refined serological differentiation of pneumococcal strains for taxonomic and epidemiological purposes

It is of special interest that all thermoresistant antigens in the model strain except the capsular polysaccharide were also present in the other strains, whether or rough This observation suggests that specific determination of anti SSS antibody in infected patients may be simplified in this way The supernate, after autoclaving and centrifugation of a suspension of the infecting strain, is used as antigen and the test serum is absorbed prior to use with a similar antigen from a rough strain The finding of trace amounts of SSS also in a rough variant, however, indicates that the rough strain employed should be controlled not to contain SSS that could interfere with the patient's antibodies

The C polysaccharide and SSS are antigens frequently employed in serological work with pneumococci, *e g* in the passive haemagglutination and complement fixation techniques It is relatively easy to prepare the C- and SSS polysaccharide antigens free from protein while separation of these antigens from each other can be difficult (9) Our finding that an electrophoretic run at pH 5 0 permitted separation of C from SSS which was not obtained at higher pH, suggests that preparative electrophoresis at acid pH could be a useful final step in purification of SSS preparations for serological work This technique might also be convenient to remove the small amounts of SSS from C polysaccharide preparations that might be present even in preparations from rough strains

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WALL TEICHOIC ACIDS IN ANIMAL *STAPHYLOCOCCUS AUREUS* STRAINS DETERMINED BY PRECIPITATION

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567 strains of *Staphylococcus aureus* isolated from a number of animal hosts were examined by agar gel precipitation for wall teichoic acids or other wall polysaccharides The species was shown to be heterogeneous with regard to these constituents Three groups could be distinguished 1 The strains which had been classified biochemically as belonging to the human bovine hare and horse biotypes, and some of the swine strains, contained polysaccharide A Of these, the horse strains contained polysaccharide Aα whereas the other strains regularly contained polysaccharide Aβ Of the latter, a higher incidence of additional polysaccharide Aα distinguished the human biotype from other biotypes 2 The dog strains contained none of the reference polysaccharides but an unknown polysaccharide (P) 3 The pigeon and mink strains contained polysaccharide C The occurrence of polysaccharide C in the biochemically least active biotypes is interesting indicating a bridge towards the polysaccharide AβC group of *S epidermidis*

The cell wall of *Staphylococcus aureus* contains a ribitol teichoic acid with either α- or β linked N acetylglucosamine residues (1,3) On double diffusion in agar gel each of the two teichoic acids gives a specific precipitation line (polysaccharide Aα polysaccharide Aβ) (16, 15 5, 19) Strains of *S aureus* either have a mixture of α- and β N acetyl glucosamine residues or only one type of linkage (26 5) These conclusions were drawn from examinations of *S aureus strains* isolated from human beings It was presupposed that animal strains of *S aureus* have the same types of teichoic acids However, animal *S aureus* strains differ considerably from human strains in their biochemical characteristics, on phage typing and on serological typing and a subdivision of the species has been advocated on this basis (24, 25, 11) In this connection information would be of interest on the type of teichoic acid present in strains isolated from various animal hosts To be of taxonomic interest a fairly large number of strains from each host would have to be examined This would not be possible by structural analysis

In the present report materials of *S aureus* strains isolated from a number of animal species have been examined for the presence of wall teichoic acids and other polysaccharides using double diffusion in agar gel with reference systems The strains had previously been characterized biochemically, by phage typing and by serological typing (see below)

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MATERIALS AND METHODS

Strains

The strains were obtained from Dr *V Hájek* and Dr *E Maršálek*, The Palacky University, School of Medicine, Institute of Microbiology, Olomouc, Czechoslovakia All strains coagulated plasma and were recorded as belonging to *S aureus* According to their animal host and the results of the bio chemical examination the strains were classified by *Hájek & Maršalek* into a number of biotypes (11) This classification has been retained in the presen tation of the materials The strains were kept in freeze dried condition Before use the cultures were tested for purity on blood agar plates

Strains isolated from cattle From different re gions of Bohemia and Moravia 91 strains were ob tained from cases of acute mastitis Seventy four strains were isolated from nasal swabs of healthy cattle from different agricultural cooperatives of the Olomouc region The results of the biochemical examination have been reported in (7) and those of phage typing, serological typing and a prelimin ary examination of polysaccharide A in (30)

Strains isolated from dogs Seventy five strains were isolated from the nasal cavities of healthy dogs from different areas of Czechoslovakia

Strains isolated from pigeons Sixty seven strains were isolated from the nasal cavities of pigeons which lived free in the town of Olomouc before capture

The results of the biochemical examination of the dog and pigeon strains have been reported in (8) and those of phage typing and serological typing in (29)

Strains isolated from swine Sixty three strains were isolated from the nasal cavities of healthy swine from different areas of North Moravia The results of the biochemical examination have been reported in (9) and those of phage typing sero logical typing and a preliminary examination of polysaccharide A in (31)

Strains isolated from hares Nineteen strains were isolated from the nasal cavities of hares killed during two winter seasons The results of the bio chemical examination have been reported in (10)

Strains isolated from mink One hundred and eight strains were isolated from mink of two farms situated in different locations of North Moravia One strain was from a septic case the remaining strains from the nasal cavities of healthy animals The results of the biochemical examination have been reported in

The report on phage typing and serological typ ing of the hare and mink strains is in preparation (*Oeding et al*)

Strains isolated from horses Seventy strains were isolated from the nasal cavities of healthy horses from different areas of Moravia and from the veterinary hospital in Olomouc The reports on the biochemical examination (*Hájek & Maršálek*) and on phage and serological typing (*Oeding et al*) are in preparation

Sera

Immune sera were produced by intravenous in jections of formalin killed bacteria into New Zea land white rabbits of the Institute's breed (27)

Double Diffusion in Agar Gel

The technique was described in (17) In the agar plate (Noble, Difco, 1 5 per cent) systems were arranged consisting of 6 peripheral (no 1 6) and one central well (no 7) 4 mm in diameter and 4 mm apart The central well was filled with un diluted antiserum and the peripheral wells with thick saline suspensions of live bacteria grown on nutrient agar Reference polysaccharide or bacteria were placed in well 1 and 4 In the polysaccharide AβC system polysaccharide Aβ was in addition placed in well 2

The plates were kept at 4° C and the reactions read daily for at least 2 days

Reference Systems

Poly Aα (poly 263)/serum *S aureus* 263 (18)
Poly Aβ (poly A)/serum *S aureus* Wood 46 (13)
Poly Bα (poly B)/serum *S epidermidis* 1254 (22)
Poly Bβ (or *S epidermidis* T2 bacteria)/serum *S epidermidis* T2 (32)
Poly AβC (or *Microc* 3519 bacteria)/serum *Microc 3519* (22)

Additional polysaccharide/antiserum systems were made from selected strains of the animal materials

RESULTS

Strains Isolated from Cattle

The results in agar gel precipitation of *S aureus* strains isolated from cattle are given in Table 1 Some of both the mastitis strains and the nose strains were found on biochemi cal examination to have the characteristics of the human biotype, whereas others were a typical and recorded as intermediate (7) In 163 of the 165 strains polysaccharide Aβ was demonstrated serologically Polysaccharide Aα was found in only six of the 144 strains of bovine biotype, whereas eight of the nine strains of human biotype contained this anti gen The two strains which lacked polysaccha ride A (one of bovine biotype, the other inter mediate), both contained polysaccharide C

TABLE 1 *Presence of Precipitinogens in S aureus Strains Isolated from Cattle*

	Biotype		Polysaccharide				
			Aα	Aβ	Bα	Bβ	C*
Mastitis 91 strains	Bovine	81	5	81	0	0	0
	Human	2	1	2	0	0	0
	Intermed	8	2	8	0	0	0
Nose 74 strains	Bovine	63	1	62	0	0	1
	Human	7	7	7	0	0	0
	Intermed	4	0	3	0	0	1

* 20 strains tested

TABLE 2 *Presence of Precipitinogens in S aureus Strains Isolated from Swine*

	Biotype		Polysaccharide					
			Aα	Aβ	Bα	Bβ	C*	V
Nose 63 strains	Porcine	47	0	22	0	0	0	2
	Others	16	11	13	0	0	0	0

* 10 strains tested

These strains are obviously of another type than the other cattle strains Biochemically and on phage and serological typing the nose strains were found to differ from the mastitis strains (30) Agar gel precipitation did not distinguish between these two groups of strains

Strains Isolated from Swine

Forty seven strains of *S aureus* isolated from the nasal cavities of healthy swine were biochemically classified as belonging to the [illegible]

In agar gel precipitation 35 of the 63 swine strains contained polysaccharide Aβ (Table 2), the frequency being definitely lower in the porcine biotype than in the other group Neither did any strain of the porcine biotype contain polysaccharide Aα in contrast to 11 of the 16 other strains

When tested in the polysaccharide Aβ system one porcine strain (V1) produced a precipitation line which gave a reaction of partial identity with a spur against the polysaccharide Aβ line Rabbit immune serum and polysaccharide material were produced from this strain and from one strain of the porcine biotype (V5) which had given no reaction in the polysaccharide Aβ system The V1 system formed a strong precipitation line in agar gel The polysaccharide involved was designated as polysaccharide V The line was demonstrated also in another porcine strain and in a few strains isolated from other animals No polysaccharide V line was observed with V5 bacteria whereas isolated polysaccharide seemed to give a faint line The possibility that other porcine strains contained small amounts of polysaccharide V could therefore not be excluded

The spur formation with the polysaccharide Aβ line indicated that polysaccharide V has a structure similar to, but not identical with, the former The inability of polysaccharide Aβ strains to react in the polysaccharide V system confirms the non identity Polysac-

TABLE 3 *Presence of Precipitinogens in* S aureus *Strains Isolated from Horses*

	Biotype		Polysaccharide				
			Aα	Aβ	Bα	Bβ	C
Nose 70 strains	Equine (canine?)	54	50	0	4	0	1
	Human	5	2	5	0	0	0
	Bovine	10	0	10	0	0	0
	Porcine	1	0	1	0	0	0

TABLE 4 *Presence of Precipitinogens in* S aureus *Strains Isolated from Dogs and Hares*

	Biotype		Polysaccharide					
			Aα	Aβ	Bα	Bβ	C*	P
Hare, nose 19 strains	Hare	16	2	15	0	0	0	0
	Canine	3	0	0	0	0	0	3
Dog nose 75 strains	Canine	63	0	0	0	0	0	52
	Human	10	6	9	0	0	0	0
	Intermed	2	0	0	1	0	0	1

* 10 dog strains and 6 hare strains tested

charide V did not cross react with polysaccharide C or polysaccharide P (see below)

The precipitation studies thus show that the strains classified as belonging to the porcine biotype are inhomogeneous with regard to their wall teichoic acids Somewhat less than 50 per cent have polysaccharide A, a few strains possibly have the unknown polysaccharide V whereas no polysaccharide was recognized in approximately 50 per cent of these strains Of the 16 strains belonging to other biotypes 13 contained polysaccharide Aβ and could be of porcine bovine or human type The frequent occurrence of polysaccharide Aα however indicates that these strains are predominantly of human origin

Strains Isolated from Horses

Fifty four of the 70 *S aureus* strains isolated from the nasal cavities of healthy horses had similar biochemical characteristics and were classified as belonging to the equine (canine?) biotype (*Hajek & Maršalek*, in preparation) The results of agar gel precipitation were very clear Of the strains belonging to the equine biotype, 50 contained polysaccharide Aα and no strain contained polysaccharide Aβ (Table 3) This pattern has not been described before in a whole collection of strains The four strains lacking polysaccharide Aα all contained polysaccharide Bα (one of them polysaccharide C in addition) These four strains, which had been shown to be atypical biochemically (*Hajek & Maršalek*, in preparation), obviously belonged to *S epidermidis* All strains which had been classified as belonging to the human, bovine or porcine biotype, contained polysaccharide Aβ and two of the human strains contained polysaccharide Aα in addition These results are in accordance with the findings presented in Table 1 and Table 2

Strains Isolated from Hares

Sixteen *S aureus* strains isolated from the nasal cavities of hares had similar biochemical

TABLE 5 *Presence of Precipitinogens in* S aureus *Strains Isolated from Pigeons and Mink*

	Biotype	Polysaccharide Aα	Aβ	Bα	Bβ	C
Pigeon nose 67 strains	Pigeon	1	1	2	0	61
Mink, nose (1 infection) 108 strains	Equine? (canine?)	0	0	0	1 (2)	108

characteristics and were classified as belonging to the hare biotype (10) Fifteen of these strains produced the polysaccharide Aβ line and two strains contained polysaccharide Aα in addition (Table 4) None of the strains of canine biotype contained polysaccharide A whereas all contained polysaccharide P (see below) Also phage typing and serological typing clearly distinguished the strains of the hare biotype from those of the canine biotype (*Oeding et al*, in preparation) Precipitation did, however, not distinguish the hare biotype strains from human bovine and a group of the swine strains all having the same wall teichoic acid

Strains Isolated from Dogs

Ten of the 75 *S aureus* strains isolated from the nasal cavities of healthy dogs belonged biochemically to the human biotype (8) The demonstration of polysaccharide Aβ and Aα in these strains substantiated the biochemical classification (Table 4) Of the two intermediate strains, one obviously belonged to *S epidermidis*, containing polysaccharide Bα

None of the 63 strains biochemically classified as belonging to the canine biotype contained any of the teichoic acids included in the test set Rabbit immune sera and polysaccharide material were therefore prepared from two strains of the canine biotype (P1, P4) A faint precipitation line shared by the two polysaccharide materials was recorded Fifty two of the canine strains and one intermediate strain were shown to have this antigen (Table 4), which was designated polysaccharide P Further, the antigen was demonstrated in the three strains of canine biotype isolated from hares (Table 4) Rabbit immune serum and polysaccharide material were produced also from one of the canine strains of hare origin (Z14) A strong precipitation line was produced which gave a reaction of identity with the line of the polysaccharide P system The Z14 system was used to test strains selected from the other materials for the presence of polysaccharide P It was demonstrated in ten out of 12 mink strains, all of which also contained polysaccharide C (see below), and in a few strains from other animals Two of these were pigeon strains neither of which had polysaccharide C

No cross reaction was observed between polysaccharide P and the other polysaccharide materials tested Preliminary investigations indicate that polysaccharide P is not a teichoic acid

Strains Isolated from Pigeons

All strains of *S aureus* isolated from the nasal cavities of wild pigeons had similar biochemical characteristics, very like those of the canine biotype (8) Pigeon strains with these characteristics have later been transferred to a pigeon biotype (11) One strain, found on agar gel precipitation (Table 5) to have both polysaccharide Aα and Aβ, was probably of human type This was consistent with the strong agglutination in serological typing and with the strain being the only one of the ma

terial typable by phage (29) Two strains containing exclusively polysaccharide Bα obviously belonged to *S epidermidis*

Of the remaining 64 strains, 61 formed a strong precipitation line which fused completely with the C line of the polysaccharide AβC system (Table 5) All these strains also formed a line against serum Wood 46 in the polysaccharide Aβ system, this line giving a reaction of partial identity with the polysaccharide Aβ line with a spur Rabbit immune serum and polysaccharide material were produced from one of these pigeon strains (H1) There was complete cross-reactivity between the H and C systems, and polysaccharide H, like bacteria from the pigeon strains produced a line with spur formation in the polysaccharide Aβ system On immunoelectrophoresis polysaccharide H and polysaccharide C gave identical precipitation lines All the pigeon strains which had produced the polysaccharide C line also produced the H line This was also true for the *"Micrococcus"* strains tested (polysaccharide AβC)

The results strongly indicate that the pigeon polysaccharide (H) is identical to polysaccharide C No cross reactivity was observed between polysaccharide C (or H) and polysaccharide V, which also reacted in the polysaccharide Aβ system with a spur formation

Strains Isolated from Mink

All the strains isolated from mink had similar biochemical characteristics and seemed to belong to the same biotype as the dog and horse strains (12) All 108 strains gave a precipitation line with a spur against the polysaccharide Aβ line and a line which fused completely with the C line of the polysaccharide AβC system (Table 5) Identity was demonstrated between the line produced by the mink strains and the polysaccharide C (and H) line

DISCUSSION

The present investigation shows that the theory of polysaccharide A being shared by all strains of *S aureus*, irrespective of host species, does not hold true There is, in fact, a wide variation within this species with regard to the types of teichoic acids and other polysaccharides found in the walls This gives room for interesting taxonomic considerations

The types of wall teichoic acids in human and bovine strains of *S aureus* have now been firmly established *Marandon & Oeding* (23), by agar gel precipitation demonstrated polysaccharide Aβ in 86 per cent of 120 bovine *S aureus* strains obtained from different sources, predominantly from cases of mastitis, and polysaccharide Aα in 5 per cent In the present materials of 154 strains classified as belonging to the bovine biotype (Table 1, Table 3) all strains except one contained polysaccharide Aβ, whereas only six strains (4 per cent) contained polysaccharide Aα The near 100 per cent incidence of polysaccharide Aβ in bovine strains parallels earlier findings in strains of human origin (14) Of the 24 strains of human biotype included in the present materials (Table 1, Table 3 Table 4), 23 contained polysaccharide Aβ There seems, however, to be a difference between human and bovine strains in the incidence of polysaccharide Aα Human strains regularly contain polysaccharide Aα in addition to polysaccharide Aβ (19), although strains in which the polysaccharide Aα line cannot be demonstrated are not unusual Of the 24 present strains of human biotype 16 contain polysaccharide Aα in contrast to only 4 to 5 per cent of bovine strains (see above) The high incidence both of polysaccharide Aβ and polysaccharide Aα among the 16 swine strains classified as not being of porcine biotype (Table 2) thus indicates that all or the majority, are of human type

Altogether, *S aureus* strains of human and bovine origin appear to be very similar with respect to their wall teichoic acids The different incidence of α glucosamine linkages is to some degree consistent with the important dissimilarities in the biochemical properties of human and bovine strains but hardly supports the establishment of a subspecies *S aureus* (var) *bovis* (24, 25)

Of the other biotypes represented in the present investigation, polysaccharide A was demonstrated also in the hare, swine and horse strains. The number of strains classified as belonging to the hare biotype is small (17), but the results of precipitation were clear (Table 4). All except one strain contained polysaccharide Aβ and two strains contained polysaccharide Aα in addition, i.e. the same pattern as in the bovine strains. This pattern was found also in 47 per cent of the strains classified as belonging to the porcine biotype. Of the remaining porcine strains, a new polysaccharide (V) was demonstrated in a few strains, whereas about 50 per cent of the strains either had an unknown polysaccharide, or no such wall material at all. The present collection of porcine biotype strains was therefore inhomogeneous with regard to cell wall polysaccharide, thus differing from the strains of the human, bovine and hare biotypes.

The demonstration of polysaccharide Aα but not polysaccharide Aβ, in all the 50 *S. aureus* strains of equine biotype (four additional strains were *S. epidermidis*) is interesting (Table 3). This pattern is clearly different from those of the other biotypes in which polysaccharide A has been demonstrated. It seems that whereas human strains usually have teichoic acid with both types of glycosidic linkages, animal strains have either α linked (horse biotype) or β linked (bovine, hare, swine (?) biotypes) glucosaminyl teichoic acid, seldom both types.

The canine and pigeon biotypes and the mink strains, differed from the other biotypes in not containing polysaccharide A. None of the 66 strains of canine biotype (three strains isolated from hares, Table 4) contained any of the teichoic acids included in the reference set. Polysaccharide material and immune serum produced from three strains of canine biotype revealed a new precipitinogen (P), which was demonstrated serologically in 83 per cent of canine biotype strains. This antigen was also demonstrated in some mink and pigeon strains.

Both the pigeon strains and the mink strains (Table 5) had polysaccharide C (22) in their walls. This precipitinogen was demonstrated in 61 out of 64 strains of pigeon biotype and in all the 108 mink strains. Polysaccharide material and immune serum produced from one pigeon strain confirmed the identity with polysaccharide C.

Strains having the two precipitinogens polysaccharide Aβ and polysaccharide C were originally described as belonging to *S. epidermidis* (22). Later the polysaccharide AβC strains were considered as micrococci (2, 28), whereas recent investigations on fundamental characteristics (*Kocur, Schleifer* & *Oeding*, unpublished) now indicate that the original classification was correct. This is in agreement with the conclusion of *Schleifer* & *Kandler* (33) and *Kocur et al.* (20) that *M. saprophyticus* and *M. lactis* should be transferred to the genus *Staphylococcus*.

The presence of polysaccharide C in animal *S. aureus* strains is interesting, indicating a bridge between *S. aureus* strains of low biochemical activity (11) and *S. epidermidis* strains of the polysaccharide AβC type, which are possibly more pathogenic than strains of the other teichoic acid types (polysaccharide Bα and Bβ) of this species (22, 4).

The nature of the new polysaccharide antigens (V, P) discovered in animal strains, and of the C polysaccharide earlier described (21, 22), is not known. Polysaccharide C has also earlier been observed to cross react with a spur formation with polysaccharide Aβ (6), and there is reason to believe that β linked glucosamine is the common determinant. Probably β linked glucosamine is also the reason for the reaction of the V polysaccharide with the polysaccharide Aβ serum. Why no reactions were observed between polysaccharide Aβ strains and polysaccharide V serum, or between the polysaccharide C and the polysaccharide V systems, is difficult to explain. Antibodies against the shared determinant might be weak in the V serum or the conditions were, for some other reason, not optimal for cross reactions. Experiments are in progress to isolate and characterize these polysaccharides.

TABLE 6 *Subspecies and Biotypes of S aureus Proposed on Biochemical Basis Compared with Types of Teichoic Acids*

Subspecies *Meyer* (24 25)	Biotypes *Hajek & Maršalek* (11)	Teichoic acid/polysacch demonstrated serologically
S aureus	A Human	Poly Aβ usually also poly Aα
	B Swine poultry	Swine poly Aβ some w poly V many w no poly demonstrated
S aureus (var) *bovis*	C Cattle sheep	Cattle poly Aβ seldom also poly Aα
	D Hare	Poly Aβ seldom also poly Aα
S aureus (var) *canis*	E Dog horse (?) mink (?)	Dog poly P Horse poly Aα Mink poly C
	F Pigeon	Poly C

An attempt to correlate the biochemical subtypes of *S aureus* proposed by *Meyer* (24 25) and *Hajek & Maršalek* (11) with the types of teichoic acids here demonstrated is illustrated in Table 6 As a general rule, polysaccharide A seems to be present in the biochemically most active biotypes, and the other polysaccharides (P, C) in the less active biotypes Thus both biochemical characteristics and the type of wall polysaccharide distinguish the dog strains from the other strains Also the biochemical classification of the mink and pigeon strains (11) agrees reasonably well with the examination of wall polysaccharide, although these strains were separated into different biotypes but contained identical polysaccharide The biochemical classification of the horse strains is not in accordance with the polysaccharide demonstrated and only some strains of the present swine biotype warrant its biochemical classification

The ICSB Subcommittee on Staphylococci and Micrococci could not recommend a subdivision of *S aureus* predominantly on biochemical criteria but considered that attention should be given to the possibility of dividing this species into varieties (34) The new information here presented of a heterogeneity of the wall teichoic acids within the species will have to be considered in connection with taxonomy In part it supports the validity of the subdivisions of *S aureus* proposed on a biochemical basis So far the presence of polysaccharide A indicates that the human bovine hare and horse biotypes and some of the swine strains belong to the same group According to the present results the dog biotype constitutes a second group and the mink and pigeon strains a third Although the strains of the present investigation were considered as independent they were all from the same country Further examinations of strains from other animal species than man and cattle, and from other geographic areas are therefore needed Basic characteristics such as the per cent GC in the DNA and the type of murein should also be examined as they can be expected to give valuable taxonomic information

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THE POTENCY OF BCG VACCINE DETERMINED BY THE TUBERCULIN SHOCK METHOD ON HAMSTERS

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BCG vaccinated hamsters, in contrast to non vaccinated, have been shown to be sensitive to tuberculin injected intraperitoneally An introductory study showed that hamsters vaccinated with a strongly potent BCG strain were killed by tuberculin shock using about the same dose of purified tuberculin as in experiments on guinea pigs. In the main experiment, hamsters were vaccinated with vaccines prepared from BCG strains of weak, medium or strong potency and injected with a large dose of tuberculin It was demonstrated that the number of animals that died of shock was significantly different in the groups vaccinated with the three vaccines Thus this method can be used for determination of the potency of a vaccine The strains examined originated from the Danish strain of BCG received from Paris in 1931

BCG vaccines prepared for clinical use in the various laboratories throughout the world are far from being uniform products The differences between them are due, to some extent, to variations in culture and production procedures, but are primarily conditioned by genotypical differences in the strains used for their preparation (*Dubos et al.* 1956) During recent years, suitable methods have been elaborated for determining quantitatively the most important biological characteristics of a BCG vaccine, *viz* its virulence allergenic potency and immunogenic potency Furthermore, a mutual positive correlation between these characteristics has been demonstrated, so that each of them can be used as indication of the potency of the vaccine (*Jespersen* 1971)

In order to assess the allergenic potency, the intracutaneous tuberculin reaction on guinea pigs vaccinated with varying doses of BCG has, in particular, been used (*Chang* 1958, *Jespersen & Bentzon* 1967) The present study deals with an attempt to achieve this by means of the tuberculin shock method on hamsters

MATERIAL AND METHODS

Experimental Animals

The hamsters were bred at the breeding farm of Statens Seruminstitut and were 2–5 months old at the commencement of the experiment The males and females were placed separately at random in guinea pig cages partitioned to house two animals Each experimental group consisted of equal numbers of females and males

BCG Strains

Dubos Copenhagen A laboratory strain from the Dubos laboratory received from Copenhagen in 1951 and sent back to Copenhagen in April 1960 Many experiments have shown that the strain has a strong lethal effect on hamsters The strain used in the present study was isolated from a hamster

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that had recently died spontaneously in a virulence experiment

Copenhagen Received from the BCG Department of Statens Seruminstitut as a freeze dried routine culture, Batch 60 produced March 17 1971

Prague Received as a freeze dried culture, Batch 725, produced May 12 1969, by the BCG Department Statens Seruminstitut, from the Prague strain

Production of Vaccine

The strains were cultured in Dubos fluid medium with Tween and subcultured at least twice before inoculation of the animals The cultures were 11 days old on the day of the experiment and had a density of 0 110 measured by Coleman spectrophotometer

Number of Bacterial Units in Vaccination Suspensions

The numbers of bacterial units in 1 ml of the undiluted suspensions, determined by inoculation of suitable dilutions on Lowenstein Jensen medium were 8×10^6 of the Dubos Copenhagen strain in the introductory study, and 12×10^6 of the Dubos Copenhagen 18×10 of the Copenhagen, and 14×10^6 of the Prague strains in the main study

Experimental

Introductory study Three groups of 20 hamsters were vaccinated intraperitoneally with 1 ml of a [illegible] he strain Dubos

injected with 10 mg [illegible] tuberculin (RT 23 without Tween) respectively The number of animals that died from shock was registered at varying times after injection of the tuberculin

Main experiment Groups of 12 hamsters were vaccinated intraperitoneally with 1 ml 10^0 1 ml 10^{-2} and 1 ml 10^{-4} culture of the Dubos Copenhagen Copenhagen or Prague strains grown in Dubos fluid medium with Tween Five weeks later all the animals were injected intraperitoneally with 10 mg purified tuberculin RT 23 That dose was chosen because a pilot study had shown that 1 mg was too small a dose to provoke shock in animals vaccinated with a weak BCG strain The number of animals that died from shock was registered at varying times from 4 to 96 hours after injection of tuberculin Observation of the animals was concluded after 10 days

Statistical analysis An estimate of the logarithm of the dose in number of viable units corresponding to the 50 per cent mortality after injection of 10 mg purified tuberculin was determined for each vaccine The method used is that described by Finney (1964), in which it is assumed that the dose response curve is logistic, and that the slope is the same for the three vaccines It should be noted that with the large intervals between the doses of vaccines used, there may be differences in the slope without this being recognized

RESULTS

Introductory Study

Determination of the smallest dose of purified tuberculin able to produce tuberculin shock None of the sixty non vaccinated hamsters died as the result of the tuberculin injection During the observation time of 7 months five died of intercurrent disease (three injected with 0 1 mg and two with 10 mg) The number of deaths from tuberculin shock among the vaccinated is shown in Table 1 In the group injected with 10 mg all the animals died—thirteen after 0–24 hours, six after 24–48 hours, and one after 48–72 hours All except three of the animals

TABLE 1 *Survival Times in Hours of Hamsters Vaccinated with Dubos Copenhagen Strain and Injected Intraperitoneally Five Weeks later with Varying Doses of Tuberculin*

Purified tuberculin RT 23		
1/10 mg 5 000 TU	1 mg 50 000 TU	10 mg 500 000 TU
♀ 24–48	♀ 0–24	♀ 0–24
♀ 24–48	♀ 0 24	♀ 0 24
♀ 24–48	♂ 0 24	♀ 0 24
Survivor	♀ 24–48	♂ 0–24
	♀ 24–48	♀ 0 24
	♀ 24–48	♀ 0 24
	♂ 24–48	♂ 0–24
	♀ 24–48	♂ 0 24
	♀ 24–48	♀ 0–24
	♂ 24–48	♀ 0 24
	♂ 48 72	♀ 0 24
	♂ 48–72	♀ 0 24
	♀ 48–72	♂ 0–24
	♀ 48 72	♀ 24–48
	♂ 72 96	♂ 24 48
	♂ 72 96	♂ 24 48
"	♂ 72 96	♂ 24–48
"	Survivor	♂ 24–48
	"	♂ 24–48
		♂ 48 72

BLE 2 *Survival Times in Hours of Hamsters Vaccinated i p with Different Doses (V U) of Prague, Copenhag and Dubos Copenhagen Strains and Injected 5 Weeks later with 10 mg Purified Tuberculin 500,000 TU*

Prague strain			Copenhagen strain			Dubos Copenhagen strain		
10^{-4} 4×10^{2}	10^{-2} 14×10^{4}	10^{0} 14×10^{6}	10^{-4} 18×10^{2}	10^{-2} 18×10^{4}	10^{0} 18×10^{6}	10^{-4} 12×10^{2}	10^{-2} 12×10^{4}	10^{0} 12×10^{6}
urvivor	Survivor	♀ 4–20	Survivor	♂ 4–20	♀ 0–4	♀ 4–20	♀ 4–20	♀ 0–4
"	"	♀ 4–20	"	♂ 20–28	♀ 4–20	Survivor	♂ 4–20	♂ 0–4
"	"	♀ 4–20	"	♀ 72–96	♀ 4–20	"	♂ 4–20	♂ 0–4
"	"	♀ 4–20	"	♀ 72–96	♀ 4–20	"	♀ 4–20	♂ 0–4
"		♀ 4–20	"	Survivor	♂ 4–20	"	♀ 4–20	♂ 0–4
"	"	♀ 28–48	"	"	♂ 4–20	"	♂ 4–20	♀ 4–20
	"	Survivor	"	"	♀ 4–20	"	♀ 4–20	♀ 4–20
"	"	"		"	♂ 4–20	"	♀ 4–20	♀ 4–20
"	"	"	"	"	♂ 4–20	"	♂ 4–20	♂ 4–20
"	"	"	"	"	♂ 4–20	"	♂ 4–20	♂ 4–20
	"	"	"	"	♂ 4–20	"	♀ 4–20	♀ 20–28
	"	"	"	"	♀ *)	"	♂ 48–72	Survivor

*) Died in the interval between vaccination and injection of tuberculin

in the group injected with 1 mg died, but the survival times were somewhat protracted in relation to the 10 mg group Only three animals in the group injected with 0 1 mg died, all after 24–48 hours, and all females

On the basis of this experiment, the suitable dose of tuberculin for use in the shock method should thus be just over 1 mg

TABLE 3 *Estimates of the Logarithm of Dose in Vable Units Corresponding to 50 per cent Mortality (LD 50) and Their Standard Errors (SE)*

BCG strain	LD 50	SE
Prague	7 18	0 27
Copenhagen	5 56	0 28
Dubos Copenhagen	4 07	0 31

Main Experiment

Tuberculin shock in hamsters vaccinated with a weak, a medium and a strong strain of BCG The number of animals that died of tuberculin shock and the survival times after injection are shown in Table 2

Twenty four of the animals vaccinated with the Dubos Copenhagen strain died, i e all except one of those given 10^{0}, all of those given 10^{-2}, and one given 10^{-4} Fifteen of the hamsters vaccinated with the Copenhagen strain died, i e all those given 10^{0} and four given 10^{-2} Only six hamsters died in the group vaccinated with the Prague strain All had been given 10^{0}, and all were females

Statistical analysis Table 3 shows the estimates and their standard error All three values are significantly different The smallest difference, strain Dubos Copenhagen versus Copenhagen, is 1 49 with a standard error of 0 42 This is significantly different from 0 ($P < 0\ 01$) The difference found between strains Prague and Copenhagen is due mainly to the difference in the number of males surviving (see Table 2)

DISCUSSION

As reported by *Hauduroy* & *Rosset* (1951 a, b), large doses of living BCG injected intraperitoneally into hamsters cause death in the course of a few weeks or months Some workers consider that under such conditions BCG may be able to multiply progressively in the organism of the hamster (*Hauduroy* & *Rosset* 1951 a, b, *Nicod et al* 1951, *Nicod* 1955, *Jespersen* 1964, *Jespersen* & *Bentzon* 1964 a) This is contradicted by others, who maintain that hamsters are no more sensitive to BCG than other animals, but that they are sensitive

to certain metabolic products or bacterial sub stances (*Berger & Puntigam* 1953, *van Deinse & Senechal* 1954, *Saenz* 1954, *van Deinse* 1956) In support of their theory, it is empha sized that both heat killed BCG vaccine and tuberculin can cause fatal disease The writer has found that heat killed BCG have a certain capacity for killing hamsters but this is clearly less than that produced by living BCG (*Jespersen & Bentzon* 1964 a) and that even in large doses, tuberculin cannot kill hamsters at all This is apparent from the introductory study, where the highest dose was 10 mg puri fied tuberculin Furthermore, the results of unpublished experiments carried out in colla boration with *Magnusson* (*Magnusson & Jespersen* 1964) showed that doses from 100 to 100 000 units of human and avian tuberculin and of sensitins produced from various atypi cal mycobacteria had no lethal effect on ham sters

In contrast to non vaccinated hamsters hamsters vaccinated with BCG vaccine are sensitive to tuberculin In a group vaccinated with a strong strain the smallest dose that was able to cause shock in almost all the ani mals was 1 mg purified tuberculin The cor responding dose in guinea pigs vaccinated with 5 mg BCG was 0 4 mg, but here the tuberculin was injected intravenously (*Friis* 1953)

The autopsy findings in hamsters that died of tuberculin shock resembled those seen in guinea pigs The lungs and organs in the ab domen particularly the liver and spleen were congested with blood and the vessels in the pleura and peritoneum were strongly dilated

Multiplication of BCG in the hamster or ganism is greater in female than in male ani mals (*Jespersen* 1964 *Jespersen & Bentzon* 1964 a) and therefore the level of hypersen sitivity sufficient for the animals to be killed by tuberculin can be expected to be reached earlier in the females This has also proved to be the case and is obvious in the group vac cinated with the Dubos Copenhagen strain and injected with 0 1 mg and 1 mg tuberculin (Table 1) and in the group vaccinated with 10^{0} of the Prague strain (Table 2

The strains examined in the main study are of special interest because they all originated from the strain that Statens Seruminstitut Copenhagen received from Paris in 1931 The laboratory in Prague began production of the Danish strain in 1947 and it was sent to Dubos in October 1951

In the present experiment the Dubos Copenhagen strain was more potent than the Copenhagen strain The same difference has been demonstrated in a virulence experiment on hamsters (*Jespersen & Bentzon* 1964 a) and in a protection experiment on red mice (*Jespersen & Bentzon* 1964 b)

The Prague strain was definitely the weak est Its poor potency has also been demon strated clearly in other animal experiments This applies both to its virulence (*Jensen & Kiær* 1957, *BCG Department Statens Serum institut Copenhagen* 1965 b) its allergenic potency (*BCG Department Statens Serum institut, Copenhagen* 1965 a) and its immun ogenic potency (*Sula* 1963) In a comparison on children of vaccines from various produc tion centres the Prague strain gave the small est tuberculin reactions and the smallest vac cination lesions (*Danish Tuberculosis Index and Statens Seruminstitut Copenhagen* 1965)

The shock method as carried out in the present study can be used to differentiate strains of varying potency even those with quite similar potency The method comprises three mutually interacting factors which must be correlated as far as possible

1) *Dose of vaccine* Instead of dilutions 10^{0}, 10 and 10^{-4}, the doses 10^{-4}, 10^{-5} and 10^{-6} have been examined However by the given vaccination time these were too small to produce an adequate level of hypersensi sitivity for the animals to be killed by 10 mg purified tuberculin A dose of 10^{-4} is too low and presumably it would be an advantage to use 10^{0} 10^{-1} 10 and 10^{-3}

2) *Vaccination time* The interval between vaccination and injection of tuberculin 11 5 weeks is probably adequate if the smallest dose of vaccine is 10^{-3}

3) *Dose of tuberculin* When comparing

vaccines one of which is as weak as the Prague vaccine, it is necessary to use a dose of 10 mg

In addition to the shock method the allergenic potency can be determined on the basis of the tuberculin reactions on guinea pigs vaccinated with varying doses of vaccine (*Chang* 1958 *Jespersen* & *Bentzon* 1967) There are no reports available where vaccines produced from different strains are compared by means of the two methods However, in experiments with guinea pigs vaccinated with Danish vaccine stored for different periods at different temperatures or with killed vaccine a positive correlation was found between the tuberculin reactions and the number of animals that died of tuberculin shock (*Friis* 1953)

The writer is grateful to *Michael Weis Bentzon* Chief of the Biostatistical Department Statens Serum Institut Copenhagen who has prepared and described the statistical analysis

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STUDIES ON A FACTOR IN INTESTINAL STRANGULATION FLUID FROM GERMFREE AND CONVENTIONAL RATS INHIBITING SERUM BACTERICIDAL ACTIVITY ON *ESCHERICHIA COLI*

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Exudate collected from a strangulated loop of intestine in conventional and germfree rats has the property of inhibiting the opsonic and bactericidal activity of normal rat serum on *Escherichia coli* Using release of radioactivity from ^{32}P labelled *E coli* as an indicator of bactericidal activity the inhibitory property of strangulation fluid on serum bactericidal activity has been subjected to further studies The inhibitory factor (FIB) resisted heat treatment for 30 min up to 100° C and did not disappear following dialysis Chromatography of strangulation fluid from germfree rats on Sephadex G 100 indicated that FIB had a molecular weight higher than 100 000 Homogenates from intestinal mucosal cells contained FIB while intestinal contents contained FIB of considerably stronger activity The results indicated that high molecular substances of intestinal origin were responible for the FIB activity of rat strangulation fluid

Exudate collected from a strangulated loop of intestine in conventional and germfree rats has the property of inhibiting the bactericidal and opsonic activity of normal rat serum in vitro, using *Escherichia coli* as test microbes (7 10) The inhibition of opsonic and bactericidal activity plays an unknown part in the complex pathophysiology of intestinal strangulation obstruction By lowering host resistance the strangulation fluid might facilitate the developement of a fatal infection The present study was performed in an attempt to study the nature and origin of the factor in strangulation fluid which is responsible for the inhibition of bactericidal activity In the following the inhibitory factor has been called FIB* As proposed by *Spitznagel* & *Wilson* (5) release of radioactivity from ^{32}P labelled *E coli* into the medium when incubated in fresh serum, was used as an indicator of the bactericidal activity of the serum used (8)

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MATERIALS AND METHODS

Animals

Germfree (GF) rats were of the CDF strain (Charles River Breeding Labs. Wilmington Mass) reared as described by *Midtvedt* & *Trippestad* (3)

*) FIB = Factor Inhibiting Bactericidal activity

Conventional (CONV) rats were of a local strain, kept under standard laboratory conditions

Strangulation Fluid

The technique used for collection of strangulation fluid from CONV rats was descibed by *Amundsen & Midtvedt* (1) The technique used in GF rats was presented in ref 10 Strangulation fluid was collected in 24 hour portions Only fluid produced later than 48 hours was used in the present experiments

The strangulation fluid was pooled, centrifuged, filter sterilized and stored at −20° C as described in detail elsewhere (6, 10) Each pool of strangulation fluid originated from at least 16 rats

Dialysis

10 ml of CONV strangulation fluid was dialyzed against 4 x 1000 ml of Krebs Ringer solution containing glucose (KRG (4)) at 4° C The dialyzed fluid was resterilized by filtration before being tested

Heat Treatment

Aliquots of 5 ml of CONV strangulation fluid filtrate were incubated for 30 min in water baths at 56° C, 80° C and 100° C respectively, followed by immediate cooling to 0° C, centrifugation at 50 000 x g for 30 min at 0° C and filter sterilization

Gel Filtration Chromatography

GF strangulation fluid filtrate was chromatographed on a Sephadex G 100 column (1 9 x 100 cm) in 5 ml aliquots The column was equilibrated and eluted with KRG of pH 7 4 at 4° C The flow rate was approximately 5 ml per hour Fractions of 1 ml were collected Each fraction was passed through a Millipore filter of pore size 0 22 μ, and stored at 20° C until tested

The column was calibrated with the following molecular weight marker substances blue dextran bovine serum albumin, ovalbumin and bovine myoglobin

Homogenization

The entire small intestine of normal GF rats was opened and the intestinal content collected The intestinal wall was washed with saline, carefully blotted dry, and the mucosal layer was scraped off using glass slides Aliquots of 1000 mg wet weigth of either mucosal scrapings or intestinal contents were homogenized in 5 ml of KRG using a Potter Elvehjem glass homogenizer The homogenates were centrifuged at 50 000 x g for 30 min The supernatant was tested for FIB activity in ten fold dilutions

Serum

Sera were pooled from CONV rats and stored at −20° C

Bacteriological Procedures

The *E coli* N7 used as test microbe was identical with the one used in other studies (3, 6, 7, 8, 9, 10, 11) The bacteria were grown, labelled with ^{32}P, and stock suspensions prepared as previously described (8)

Preincubation and Preparation of Test Media

One volume of strangulation fluid filtrate, 7 volumes of KRG and one volume of serum were mixed in that sequence at 0° C In other experiments the strangulation fluid filtrate was replaced by tissue homogenates, prepared as described above Test media containing fractions collected at gel filtration were prepared as follows to 8 volumes of each fraction was added one volume of serum Control media consisted of 8 volumes of KRG and one volume of serum

Each medium was preincubated at 37° C for 60 min, then immediately cooled to 0° C To each of the preincubated media was added one volume of the stock suspension of bacteria The final test medium contained 10^9 labelled bacteria per ml, 10 per cent serum and 10 per cent of either strangulation fluid filtrate or homogenate of appropriate dilution The fractions from gel filtration were not diluted

Determination of Release of Label from Bacteria into the Medium

Aliquots of 2 ml of the suspension of ^{32}P labelled *E coli* into the medium to be tested, were incubated at 37° C for 15 min The suspension was immediately cooled to 0° C and centrifuged at 6000 x g for 10 min at 0° C The radioactivity released into the supernatant was determined by liquid scintillation spectrometry, as described in detail elsewhere (8) Results are presented as per cent inhibition, i e

$$100 - \frac{S}{C} \times 100$$

S cpm of sample in question
C = cpm of control

Statistics

The two-sample ranks test of Wilcoxon White was used (2)

RESULTS

It appears from Fig 1 that heat treatment of CONV strangulation fluid filtrates up to 100° C for 30 min did not diminish the FIB

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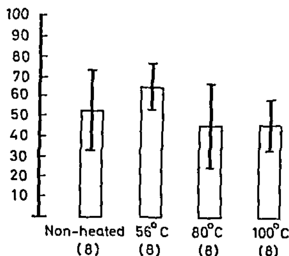


Fig 1 FIB activity of CONV strangulation fluid filtrate, heated for 30 min at 56° C, 80° C and 100° C
Each column represents the mean of eight observations (two experiments) ± one standard deviation

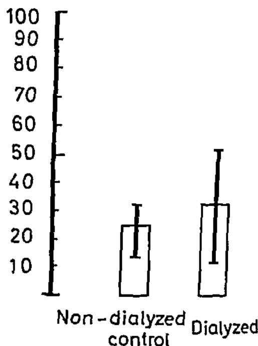


Fig 2 FIB activity of CONV strangulation fluid filtrate following dialysis for 24 hours
Each column represents the mean of 10 observations (three experiments) ± one standard deviation

activity Following heat treatment at 56° C, the inhibitory activity seemed higher than that of the nontreated strangulation fluid filtrate The difference was not statistically significant, however ($p>0\ 10$)

The inhibition of bactericidal activity in · ced by dialyzed CONV strangulation fluid filtrate was 43 per cent, while that of the non dialyzed filtrate was 27 per cent (Fig 2) The higher FIB activity following dialysis was not statistically significant ($0\ 10> p<0\ 05$)

To diminish effects of bacteria and enzymes during chromatography, and avoid confusion with bacterial products, strangulation fluid from GF rats was used, and separation carried out at 4° C When the fractions were tested FIB activity was demonstrated only in the high molecular region, with a peak corresponding to void volume (Fig 3)

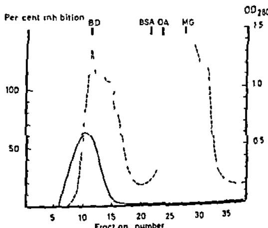


Fig 3 Gel filtration on *Sephadex* G 100 of strangulation fluid filtrate from GF rats
Per cent inhibition of bactericidal activity ——
OD_{280} – – –
The figure illustrates results from a *single experiment* *Three experiments were performed with* consistent results
Abbrevations BD = blue dextran BSA = *bovine* serum albumin OA = *ovalbumin* MG = myoglobin

In an attempt to establish the origin of FIB in strangulation fluid homogenates of intestinal mucosa and intestinal contents from normal *GF* rats were tested for inhibition of bac-

TABLE I *Per Cent Inhibition of Bactericidal Activity Induced by Homogenates of Normal GF Rat Tissues*

Final concentration (wet weight per ml)	20 mg	2 mg	0 2 mg
Intestinal contents	85 ± 3 (11)	69 ± 7 (11)	18 ± 4 (8)
Intestinal mucosa	66 ± 14 (12)	0 (12)	0 (8)

The results are the mean of three different experiments ± one standard deviation
The numbers in brackets represents the numbers of observations

tericidal activity As shown in Table I intestinal contents in concentrations of 2 mg per ml induced more than 60 per cent inhibitory activity while 20 mg per ml was reqired of the mucosal homogenate to produce FIB activity of the same degree

DISCUSSION

Previous studies have shown that sterile filtrates of strangulation fluid from rats inhibit the opsonic and bactericidal activity of serum on *E coli* (7 10) The nature of the substances involved has not been established FIB appeared to originate from the animal itself and not from the intestinal bacterial population since strangulation fluid from GF rats contained FIB activity which was equally or even more potent than that of strangulation fluid from CONV rats (10)

To simplify technical procedures estimation of release of label into the medium was chosen as test method of serum activity This method is less time consuming and simpler to perform than measuring opsonization by means of phagocytes (8) FIB showed marked heat stability Heating for 30 min up to 100° C did not abolish FIB activity Strangulation fluid heat treated at 56° C appeared to induce stronger inhibition of bactericidal activity than did non treated fluid Although the results were not statistically significant further proof might be added from the observation that samples of strangulation fluid which did not show FIB activity did so following heat treatment at 56° C (Unpublished results)

Previous results have indicated that strangulation fluid filtrates together with FIB a[ctivity] contain a factor with the opposite effect i e a factor which enhances the bactericidal activity of serum (9) Enhanced FIB activity in strangulation fluid heat treated at 56° C might be due to inactivation of components in the fluid stimulating serum bactericidal activity

FIB was not removed from the filtrate by dialysis The possibly enhanced FIB activity following dialysis might be due to removal of substances in the filtrate which stimulate the bactericidal effect of serum

Being non-dialysable, FIB seemed to consist of large molecular substances In an attempt to determine molecular weight more accurately, chromatography was carried out using Sephadex G 100 Strangulation fluid from GF rats was used for two reasons 1) to eliminate bacterial products 2) because strangulation fluid from GF rats produced more pronounced FIB activity than did strangulation fluid from CONV rats (10) The peak of inhibitory activity was found in fractions which corresponded to void volume This indicate molecular weights exceeding 100 000

FIB activity was demonstrated in intestinal contents from normal GF rats as well as in homogenates of intestinal mucosa of the same animals The results indicated that the FIB activity of intestinal contents was at least

ten times more potent than that of intestinal mucosa Based upon these findings, it might be reasonable to assume that FIB is a product of intestinal mucosa, secreted to and concentrated in the intestinal contents

The results of the present study indicated that macromolecules of intestinal origin were responsible for inhibition of bactericidal activity The heat stability of FIB indicated that the substances were hardly of pure protein nature Further studies on extracts from intestinal contents and various intestinal tissues will be presented in another communication (11)

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RABBIT ANTI RAT LYMPHOCYTE SERUM: IN VITRO ACTIVITY TOWARDS MACROPHAGES IN MONOLAYER

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A method for demonstration of cytotoxic antibodies in antilymphocyte serum (ALS) towards macrophages is described. Peritoneal macrophages were cultured in a medium containing ^{3}H Leucine for 16 hours. Substitution of the culture medium with a medium containing ALS and active complement, resulted in rapid release of radioactivity into the medium, compared to the slow spontaneous release in control experiments. The release of label was dependent on active complement.

It is generally believed that the immunosuppressive effects of antilymphocyte sera (ALS) are mediated by their effect on lymphocytes (7, 16). In vivo effects of ALS on macrophage function and on the activity of the reticuloendothelial system have been demonstrated by many (1, 3, 8, 13, 14). The presence of in vitro activity towards macrophages has, however, been difficult to assess. Many workers using various types of ALS have been unable to demonstrate such activity (2, 3, 16). It has, however, recently been demonstrated that ALS may cause aggregation of single macrophages in suspension (10), and increase cytoplasmic contact between macrophages while in culture (9). These test systems were unaffected by complement.

The present study describes a method of testing in vitro cytotoxic activity of ALS on macrophages. Addition of antiserum and complement to pure and viable labelled macrophages in monolayer resulted in release of radioactivity into the medium.

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The method was described at the V th meeting of the European Society for Experimental Surgery in Venice Italy 11th–13th May 1970.

MATERIAL AND METHODS

Preparation of Antisera

Rat spleen cells were obtained as previously described (5). Rabbits were immunized intravenously with 0.8–1.4 × 10^8 viable spleen cells twice weekly for 4 weeks. The rabbits were bled by heart puncture 10 days after the last immunization. Antiserum and normal rabbit serum were inactivated by heating to 56° C for 30 min prior to storage at –20°C. The sera were identical to some of those described earlier (5).

Macrophage Monolayer Technique

Mononuclear cells were obtained from rats injected intraperitoneally with sodium caseinate. Sodium caseinate (Nutritional Biochemical Co. Cleveland, Ohio) was dissolved in 0.15 M NaCl solution to a final concentration of 12 per cent (w/v). The solution was sterilized by autoclaving at 120° C for 30 min and stored at 4° C until used. 3 days after intraperitoneal injection of 20 ml sodium caseinate solution, the rats were an

aesthetized with ether and 20 ml sterile Hanks solution with 10 IU heparin/ml was injected into the peritoneal cavity After gentle massage the abdomen was opened, and the fluid pipetted into sterile tubes kept in ice water

The cells were washed three times in chilled Hanks' solution before being suspended in Puck's medium Following staining with methyl violet the cells were counted in a Burker's chamber The differential count invariably showed 30 40 per cent macrophages, the rest consisted of polymorphonuclear leucocytes and lymphocytes

Aliquots of 1 ml of cell suspension containing 1 1 5 x 10^6 macrophages per ml were incubated at 37° C for 60 min in tissue culture tubes with a 6 6 cm^2 flat bottom (Bellco Glass Inc Vineland, N J) The medium, containing dead and nonattached cells was then removed by decanting and the monolayer of attached viable macrophages was washed in 2 ml sterile Hanks' solution After washing, the monolayer was labelled with ^{3}H Leucine ^{3}H Leucine was supplied as L Leucine in sterile solution (Institutt for Atomenergi Kjeller Norway) The spesific activity was 1 μCi/ml The isotope solution was diluted 1 20 with sterile 0 15 M NaCl before use The macropage monolayer was covered with 1 ml Puck s medium containing 10 μl isotope solution, and incubated at 37° C After 16 18 hours incubation the medium containing dead and detached cells was removed by decanting The cell layer attached to the glass wall was then rinsed by tilting the tubes 10 times in three changes of 2 0 ml sterile Hanks solution The macrophage monolayer was then ready for testing of release of label In some experiments cells were cultured on sterile cover slips At the termination of the experiment, the coverslip was rinsed and the cells fixed in ethanol and stained with Weigert s stain and examined with light microscopy

Testing Release of Label

The antimacrophage activity of the different antisera was studied by adding Puck s medium with antiserum and complement to the labelled macrophage monolayer The effect of the antiserum on the macrophage monolayer was evaluated by measuring the amount of radioactivity released into the medium

Three parallell culture tubes were used for each serum dilution Each experiment contained control tubes with guinea pig serum (frozen and once thawn) and medium Tubes showing signs of infection indicated by change in pH and resultant colour change, were discarded

Doubling dilutions of 0 25 ml inactivated antiserum or normal rabbit serum were made in Puck s medium, 0 25 ml guinea pig serum and finally 0 5 ml Puck s medium was added to make the volume 1 0 ml per tube In some experiments ALS was added without complement and in others heat inactivated complement was added

Aliquots (100 μl) of test or control media for estimation of the zero radioactivity were taken within 1 min of adding the test or control medium to the culture tubes The tubes were then placed horizontally at 37° C until the next aliquot was withdrawn Aliquots were withdrawn every 30 min for 2 hours In some incubation experiments the medium being 2 5 ml per tube aliquots were also withdrawn at 4, 6, 8 and 24 hours Aliquots removed for counting of radioactivity were pipetted directly into scintillation vials to which 10 ml of scintillation fluid was added (400 g naphtalein, 25 g 2 5 diphenyloxazole, 0 25 g 1 4 di (2 (5 phenyloxazolyl)) benzene, 1925 ml dioxan 1925 ml xylol and 1150 ml absolute alcohol) In some experiments test media were tested for radioactivity before and after passage through a 0 22 μMillipore filter

At the end of each experiment the radioactivity of the cell residue was measured The remainder of the medium was decanted and the monolayer washed once in 2 0 ml Hanks solution To measure the radioactivity of the cell residue, 1 0 ml Hyamin (Hyamin hydroxide (1 0 M solution in methanol) Koch Light Lab Ltd , Colnebruch Buchs England) was added to each culture tube The tubes were then corked and vigorously shaken for 1 min, followed by incubation for 30 min at 37° C Aliquots were then withdrawn for determination of residual radioactivity Radioactivity was determined in a Nuclear Chicago Mark II liquid scintillation counter at 8° C

The release of label (ROL) in per cent from the monolayer was expressed accordingly

$$\frac{\text{cpm of medium at a given time} - \text{cpm of the medium at zero time}}{\text{cpm total (increase in cpm of medium + cpm of cell residue)}} \times 100$$

RESULTS

Light microscopy studies of the cells on the coverslips revealed cells of a uniform type staining identically with a faint basophilic cytoplasma and a round frequently indented nucleus Most of the cells were elongated with well developed pseudopodia No granulocytes were seen (Fig 1)

Spontaneous release of radioactivity was found in the presence of active complement and followed a consistent pattern (Fig 2)



Fig 1 Light microscopy of macrophage monolayer at end of incubation period Weigert's stain Note uniformly staining cells and development of pseudopodia

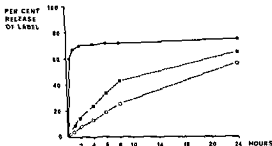


Fig 2 Release of radioactivity from the macrophage monolayer during 24 hours Comparison of spontaneous release into medium containing only complement (control) – o – o – with release into medium containing inactivated normal rabbit serum and complement – ■ ■ —— ■ – and with medium containing antilymphocyte serum and complement —— ● —— ●

Addition of inactivated normal rabbit serum and complement to the medium resulted in slightly more release of label, but the release curve followed a similar pattern Within the first 2 hours there was a gradual release of label always less than 20 per cent of the total incorporated activity The release gradually increased within the first 8 hours, when a levelling off was observed

When rabbit anti rat lymhocyte serum and complement was included in the medium, a marked change occurred (Fig 2) The maximum release was observed within the first 2 hours The total release of label throughout a 24 hour period was, however, only slightly greater than that observed with normal rabbit serum, or with guinea pig serum alone

The ROL system was complement dependt, as illustrated in Fig 3 Antiserum alone or antiserum with inactivated complement showed the same release of label as did the controls Release with inactivated

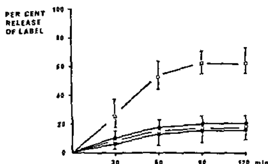


Fig 3 The effect of active complement and antilymphocyte serum (ALS) on the release of radioactivity from a macrophage monolayer Each point represents mean of three single culture tubes Vertical bars indicate range of single results. —— ◻ —— ALS with complement —— ■ —— ALS without complement, —— o —— ALS with heatinactivated complement, —— ● —— normal rabbit serum with complement

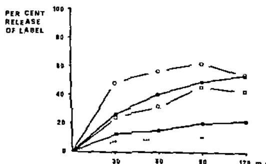


Fig 4 Release of radioactivity from labelled macrophage monolayers The effect of different concentrations of antilymphocyte serum (ALS) and standard dose complement in relation to period of incubation Each line represents mean results of three single culture tubes —— o —— o ALS 1/8 —— ● —— ● ALS 1/128 —— ◻ —— ALS 1/256 —— ◻ —— ◻ ALS 1/512 control

normal rabbit serum was the same as in the control experiments (Fig 3) The means of the control tubes always showed less than 20 per cent release of label even after 2 hours (*Fig 3*)

ROL from individual single tubes did not show a large degree of variation (Fig 3) The ROL test gave reproducible results, successive experiments carried out using the same antiserum, resulted in nearly identical release curves

Radioactivity of the medium at 90 min was not lowered after passage of the medium through a 0 22 μ Millipore filter (2160 cpm versus 1900 cpm, 1290 cpm versus 1290 cpm) Thus the increase in radioactivity was not due to dead, detached cells (with in*corporated radioactivity) floating in the* medium

Fig 4 illustrates the release curves with varying concentration of ALS Dilution of ALS 1/8, 1/128, 1/256 and 1/512 are shown The release curves for the three first dilutions are fairly identical, though with a tendency to less release with increasing dilution *Dilution of the antiserum from* 1/256 to 1/512 resulted in marked change in the release curve With the highest dilution, the release curve nearly paralleled the curves obtained from the control tubes With a dilution of 1/256 a maximal ROL of 45 per cent was reached while with a dilution of 1/512, the maximal ROL was approximately 20 per cent

The ROL titre of an antiserum could thus be defined as the reciprocal of the final dilution of the antiserum showing more than 40 per cent release of label which is twice the release obtained with normal rabbit serum within 2 hours

The Figure furthermore illustrates that a plateau was reached within 60–90 min incubation with all serum dilutions (see also Fig 2) This indicates that to assess the antimacrophage activity of an unknown antiserum only two samplings of the medium should be sufficient In addition to the zero sample the optimal time for the second sampling is after 90 min incubation

DISCUSSION

The ROL (release of label) test was designed to investigate the effect of an anti serum on pure and viable macrophages This was achieved by letting the macrophages incorporate radioactivity during a period before the testing The test was carried out on macrophages in monolayer, and not on single detached cells The test measured cytotoxic activity as indicated by the dependency on active complement The cytotoxic damage presumably resulted in leakage of intracellular labelled components similar results have been obtained with labelled bacteria (15) This is further supported by the fact that at least 50 per cent of the label in the medium was bound to proteins and *peptides, as indicated by precipitation* of the medium by trichloracetid acid (*Jakobsen,* unpublished observations) The results were consistent and reproducible

According to the evidence put forward by *Hirsch et al* (4) the release of radioactivity into the medium could be due to induced lack of adhesiveness, inhibition of attachment and *resultant flotation of cells* into the medium Filtration of medium through a Millipore filter did, however not diminsh the activity in the medium The test thus seemed to measure the release from attached living cells

Previous studies show conflicting evidence on the antimacrophage activity of ALS Many workers have shown that ALS may affect the reticulo-endothelial system with impaired intravascular clearance of colloidal carbon (*3, 8 14*) and gelatinous lipid emulsions (13) Direct injury to macrophages, has been suggested as a possible cause (*1, 13 14*)

Evidence for in vitro activity of ALS towards macrophages is conflicting Various techniques have been used to assess anti macrophage activity and *different* preparations of ALS have been tested Neither *Woodruff et al* (16) using an anti thoracic duct cell ALS nor *Gill and Gotjamonos* (3) using an antilymphnode ALS nor *Dyminski and Argyris* (2) testing an anti spleen cell ALS could find any in vitro cytotoxic ac

tivity of their ALS towards macrophages Mixed peritoneal cells consisting of macrophages lymphocytes and granulocytes were used as target cells and the effect was measured in a dye exclusion system with Trypan Blue A mixture of different cell types as target cells is not ideal as the concomitant and perhaps competing effect on other cell populations (e g lymphocytes) may influence the results Furthermore dyes such as Trypan Blue stains the cytoplasma of living macrophages which may thus be confused with the staining of dead cells (11)

Cultured macrophages have previously been used to assess antimacrophage activity In order to obtain single cells the macrophages have been detached from the culture glass wall either mechanically or by trypsin treatment (8 10) Only slight antimacrophage activity could be demonstrated by *Loeuı et al* (8) It may be that the mechanical detachement resulted in some cell damage Trypsin treatment have possibly resulted in inability of the cells to fix complement as no cytotoxicity only agglutination could be demonstrated (10) In a tissue culture monolayer *MacLaurin and Humm* (9) recently demonstrated an increase in cytoplasmic contacts with subsequent aggregation between macrophages on exposure to anti thymus ALS This effect was however unaltered by complement

The demonstration of antimacrophage activity of ALS seems primarily to be dependet on a test system which is specific for antimacrophage activity The present findings are in keeping with this They further support and extend the observations by *Marsman et al* (10) and *MacLaurin and Humm* (9) by demonstrating in vitro cytotoxic activity Variances in the nature of the antigen used and the immunization protocol may however also be of great importance The influence of some of these factors have been investigated separately (6) indicating that antimacrophage activity of ALS may be more common than thought previously

The practical implications and the possible in vivo activity of this antimacrophage effect is not known It has been suggest that the antimacrophage activity may play a part in determining the immunosuppressive activity of ALS (9, 14) Antimacrophage antisera have been shown to exhibit some immunosuppressive activity (2, 12) Using the present test system a negative correlation between ROL activity and ability to prolong the survival of skin allografts in rats does however, seem to exist (6)

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RABBIT ANTI RAT LYMPHOCYTE SERUM: IN VITRO ANTIMACROPHAGE ACTIVITY OF DIFFERENT TYPES OF ANTISERA AND RELATIONSHIP TO IMMUNOSUPPRESSION

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Antimacrophage activity has been demonstrated in antilymphocyte sera raised with thoracic duct, thymus and spleen cells Comparison of various anti spleen cell antisera raised with different antigen doses (1×10^6 – 1×10^9 cells) and immunization schedules (2, 3, 4, 6 and 8 immunizations) was carried out The presence and degree of antimacrophage activity was related to the cell dose and even more so to the number of immunizations Antisera with strong antimacrophage activity were less able to prolong the survival of skin allografts than antisera with only moderate antimacrophage activity, the lymphocytotoxic titres being similar Possible mechanisms for the presence of antimacrophage activity in antilymphocyte sera and in vivo implications of such activity are discussed

In a previous report a method for detection of antimacrophage activity of antilymphocyte sera (ALS) has been described (9) The present paper demonstrates antimacrophage activity of antisera raised with different types of antigen and various immunization schedules The activity towards macrophages has been compared with the lymphocytotoxic activity and with the ability of some of these antisera to prolong the survival of skin allografts in rats

MATERIALS AND METHODS

Preparation of Antisera

Rabbit antisera were raised with three different cell types Rat spleen and rat thymus cells were obtained as previously described (7, 10) Thoracic duct lymphocytes were obtained from rats weighing 150–200 g as described by *Bollman et al* (2) Free flow of lymph was obtained in each case and no evidence of clot formation or active bleeding was noticed Lymphocytes were collected during the first 24 hours after cannulation Viability counts, using Trypan Blue, invariably showed 90–95 per cent viable cells

Rabbits were immunized intravenously with either thoracic duct, thymus or spleen cells according to the immunization schedule (Table 1) "3 pulse" antisera were raised with one weekly immunization, all other antisera with two weekly immunizations Some of these antisera have been described earlier (7, 8) Serum was collected 10 days after the last immunization, and inactivated by heating before storage at –20°C

Macrophage Monoplayer Technique

This was carried out as described in detail previously (9) Aliquots were withdrawn from the incubation medium at zero time, after 90 *min in* cubation and from the cell residue for determination of radioactivity The ROL (release-of label)

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titre was defined as the reciprocal of the final dilution of antiserum giving at least 40 per cent release of label

Lymphocytotoxic Test

This was done as previously described using Trypan Blue, guinea pig serum as complement and blood lymphocytes as target cells (7)

Skin Grafting Procedure

Full thickness skin grafts were transplanted from inbred male Fischer to inbred male hooded BDE rats. A total of 22 ml of ALS was given subcutaneously from day −7 to day +7 The allograft survival data have been published previously (8)

RESULTS

The in vitro activity of the different antisera is given in Table 1 Both antisera raised with thoracic duct cells showed antimacrophage activity as did both antisera raised with thymus cells The individual antisera varied in activity by up to one dilution The anti thymus ALS showed slightly more activity than did the thoracic duct antisera, the lymphocytotoxic titres being about equal

No antimacrophage activity could be detected in antisera raised with 1×10^6 spleen cells, employing 8 immunizations. Neither was any antimacrophage activity seen in antisera raised with 1×10 spleen cells employing 2 or 4 immunizations while 8 immunizations resulted in strong ROL titres (64 and 128) These antisera showed considerable lymphocytotoxic activity, with titres ranging from 32–512

Raising the antigen dose to 1×10^8 spleen cells resulted in antimacrophage activity of antisera raised with only 3 immunizations similar to the results obtained with thymus and thoracic duct cells The lymphocytotoxic titres were significantly higher The antimacrophage activity of these antisera was, however, less (by one double dilution) than antisera raised with 1×10^7 cells and 8 immunizations Eight immunizations with 1×10^8 spleen cells increased the antimacro-

TABLE 1 *Type of Antigen, Immunization Schedule and in Vitro Antimacrophage and Antilymphocyte Activities of Different Antisera*

Antigen	No of cells	No of immunizations	ROL titre*	Lymphocytotoxic titre*
Thoracic duct cells	1×10^8	3	16	128
	1×10^8	3	16	256
Thymus cells	1×10^8	3	32	256
	1×10^8	3	64	256
Spleen cells	1×10^6	8	< 8	64
	1×10^6	8	< 8	128
Spleen cells	1×10	2	< 8	32
	1×10^7	2	< 8	64
Spleen cells	1×10^7	4	< 8	128
	1×10^7	4	< 8	512
Spleen cells	1×10	8	64	512
	1×10^7	8	128	1024
Spleen cells	1×10^8	3	32	512
	1×10^8	3	64	1024
Spleen cells	1×10^8	8	256	1024
	1×10^8	8	256	2048

* Antimacrophage activity measured as ROL (release-of label) from labelled macrophage monolayer antilymphocyte activity measured as cytotoxic activity with dye exclusion

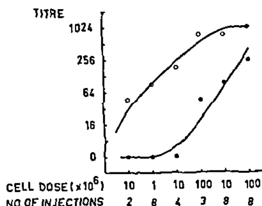


Fig 1 The relationship between ROL titres and lymphocytotoxic titres of antilymphocyte sera raised with spleen cells with the antigen dose (number of cells) and number of immunizations ROL titres indicated by ● lymphocytotoxic titres by ○ The lines have been drawn only to indicate that a systematic development of antibodies with activity both towards lymphocytes and towards macrophages seems to exist with a dependency on the immunization schedule

phage activity by 2–3 double dilutions, compared to "3 pulse" ALS Little, if any concomitant increase in the lymphocytotoxic activity was observed

The lymphocytotoxic titre and the ROL titre of each type of antispleen ALS have been related to the immunization schedule (Fig 1) Development of antilymphocytic and antimacrophage activity depended on the cell dose and the number of immunizations Employment of a certain immunizing dose of spleen cells was necessary before antimacrophage activity became detectable, though antibodies to lymphocytes were readily demonstrable With increasing number of immunizations, the antimacrophage activity increased sharply, eventually without any concomitant increase in lymphocytotoxic activity

The immunosuppressive effects of two different pools of antispleen cell ALS on survival of skin allografts and the anti macrophage activity of these antisera are shown in Table 2 Other in vitro characteristics of the two *pooled antisera, "3 pulse" and "8 pulse" ALS* have been published in a previous report (8) "3 pulse ALS was shown to increase the allograft survival from 12 0±0 45 to 21 1±0 77 days, while the "8 pulse" ALS only prolonged the survival to 16 0±1 48 days The ROL activity of the two types of antisera differed by 3 double dilutions, while the *lymphocytotoxic titre differed by one double* dilution (Table 1) The immunosuppressive effect of antisera with a moderate ROL activity were superior to antisera with a strong ROL activity An inverse correlation between the ROL titre and immunosuppressive effect thus seemed to exist

TABLE 2 *Relationship between ROL Titre, Lymphocytotoxic Titre and Ability to Prolong Survival of Skin Allograft in Rats of Two Different Pooled Batches of Antilymphocyte Sera Raised in Rabbits with Spleen Cells (1×10^9 Cells per Immunization)*

Type of treatment	ROL titre	Lymphocytotoxic titre	Mean ± S D	Student T test.
Untreated			12 0 ± 0 25	
Normal rabbit serum	< 8	8	10 0 ± 0	
3 pulse ALS	32	1024	21 7 ± 0 77	$p < 0\,001$ versus untreated; $p < 0\,001$ versus "8 pulse"
8 pulse ALS	256	2048	16 0 ± 1 48	$p < 0\,001$ versus untreated

Each experimental group consisted of 10 animals, apart from the normal rabbit serum group which only consisted of 5 animals

23*

DISCUSSION

In vitro antimacrophage activity has been demonstrated in antisera raised with either thoracic duct cells, thymus cells or spleen cells The different spleen cell antisera demonstrated that this activity was related both to the cell dose and even more to the number of immunizations Antispleen cell ALS with relatively strong immunosuppressive activity only contained antimacrophage activity of moderate strength, while antisera with a strong activity towards macrophages showed less ability to prolong the survival of skin allografts

The antimacrophage activity of spleen cell antisera could be explained by the presence of macrophage in the inoculum The antimacrophage activity of the thoracic duct cell antisera and of the anti thymus antisera require a different explanation, though the presence of monocytes or possibly macrophages among the cell population of the thoracic duct cannot be ruled out By processing the thymus gland, macrophages could have become included among the inoculum cells Screening to avoid such contaminating cells of the inoculum was not carried out Development of antibodies reacting with both lymphocytes and macrophages is the most likely explanation This explanation may also be relevant, at least partly, for the spleen cell ALS

Development of antibodies reacting with other cells than the inoculum cell has been demonstrated previously Immunizing rabbits with pure human lymphocytes *Thorsby* (21) obtained lymphocyte specific antibodies and in addition antibodies reacting both with lymphocytes and granulocytes Similarly *Marsman et al* (18) testing rabbit anti guinea pig ALS raised with pure lymphocytes demonstrated specific antibodies to lymphocytes and common antibodies to lymphocytes and macrophages The specificity of the antibodies of the present study was not determined Human fibroblasts are known to exhibit HL-A specificities (22) and antisera raised with fibroblasts have been shown to exhibit in vitro lymphocytotoxic activity and immunosuppressive activity in vivo (15) On the basis of these experiments, the existence of common antigens between the respective cell types has been postulated (15, 18, 21) *Unanue* (23) using rabbit antisera raised with cultured mouse macrophages could, however, not demonstrate crossreactivity

The production of antibodies with strong affinity for one cell type such as lymphocytes with concomitant, but weaker affinity for another cell type such as macrophages, may be another explanation Because of similarity, though not identity between antigenic determinants, such antibodies would be able to cross react with cell types other than lymphocytes

An important question is, however, whether such cross reactivity indicated by a high ROL titre adds to or diminishes the immunosuppressive potency of an antiserum As macrophages are thought to play a part in the immune response, by processing or presenting the antigen material to the lymphoid system modification of this process may possibly result in immunosuppression Antimacrophage antisera have indeed been reported to suppress antibody formation (19) and to prolong survival of skin allografts (4) These antisera may, however, have contained antilymphocytic activity in addition to antimacrophage activity, as the antisera were raised using peritoneal cells, containing some 10–25 per cent lymphocytes In contrast, *Loewi et al* (16) found that a pure rabbit anti rat macrophage antiserum was unable to suppress the antibody response to sheep red blood cells neither could *Land et al* (14) demonstrate any increased survival of skin allografts in rats with a pure antimacrophage antiserum, raised in rabbits During the production of this antiserum, the rabbits were given rabbit anti rat ALS in order to suppress formation of specific antilymphocyte antibodies

The findings of the present study did not indicate that in vitro antimacrophage activity contributed to the immunosuppressive activity of the antispleen cell ALS On the contrary strong antimacrophage activity seemed to diminish the immunosuppressive effect Thus the broadening of the antibody specificity

whatever its cause, may lead to a decrease in the specific antilymphocytic effect (Fig 1), and thus signal a decrease in the in vivo potency of the ALS Two recent publications suggest this By varying the antigen dose from 1×10^6 to 1×10^9 cells and the immunization schedule, *Gozzo et al* (5) showed that high dose, 'multiple pulse' immunization schedule leads to a sharp increase in antibody titre towards erythrocytes and serum proteins with a concomitant weakening of the immunosuppressive effect No direct comparison between 'irrelevant' antibody activity and immunosuppressive activity was however, performed Similarly, *Rossen et al* (20) were unable to demonstrate any in vivo immunosuppressive activity of antisera with concomitant strong activity towards erythrocytes, platelets and glomerular basement membrane

Many workers have demonstrated that antisera raised with few immunizations have better immunosuppressive properties than those raised with multiple immunizations (1, 3 8) No definite explanation has been given *Levey & Medawar* (15) have suggested that this may be due to either a 'change in physical character of the operating antibodies' or because of interference by antibodies to minor or irrelevant constituents of the immunizing cell

It has previously been demonstrated that during a multiple immunization schedule, there is a change in the lymphocytotoxic antibodies from IgM to IgG (7) The most potent in vivo sera contained much of the in vitro activity in the IgM fraction Although most workers have found the bulk of the immunosuppressive effect of different antisera in the IgG fraction (6 12 13 17), immunosuppressive effect of the IgM fraction has been definitely demonstrated (10 11, 17) The change from IgM to IgG antibodies could therefore at least partly explain differences in immunosuppressive properties of different antisera This is however probably not the whole explanation The development of antibodies with broadened specificity (such as activity towards macrophages in vitro) and with concomitant loss of specific antilymphocytic and thus immunosuppressive activity may be an additional explanation

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A MICRO ASSAY FOR MOUSE AND HUMAN INTERFERON.

II Dose-Response in Different Cell/Virus Systems

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In a micro assay of mouse and human interferon a straight line relationship is demonstrated between the dose of challenge virus and the resulting interferon titre The dose response regression lines are calculated for a variety of cell/virus/interferon systems and the slopes of the regression lines are shown to depend on the challenge virus used while the interferon and the cell system have only minor influence if any

Calculation of interferon titres implies a correlation between the virus effect in the controls and the effect in the test units In some test systems the correlation can be done directly by meassuring the degree of inhibition of viral effect compared to the controls as in the plaque reduction method (6 15), the yield reduction method (4, 5), the hemagglutination inhibition method (3, 14) or methods based on the inhibition of viral nucleic acid synthesis (1, 9 16) In infectivity inhibition assays however, there is no such built in correlation as the titre of interferon is defined as the dilution, which inhibits the viral cytopathogenic effect (CPE) in 50 per cent of the test units when challenged against a prefixed standard dose of virus, i e 10 or 100 tissue culture infectious doses ($TCID_{50}$) As small variations in virus titre easily may take place influencing the reproducibility of the test considerably (11), a minute virus titration must be run simultaneously with eac[h] test But still there is a need for a calibratio[n] curve relating the dose of challenge virus use[d] to the corresponding interferon titre

In a previous study on a micro assay [o]f interferon (2) a straight line relationship wa[s] found between the dose of challenge viru[s] used and the resulting interferon titre bot[h] expressed in $\log_{10}$ units, when human inter[-]feron was assayed in the U cell/Vesicula[r] Stomatitis virus system The slope of the re[-]gression line was identical with that found when mouse interferon was tested in the L-[F] cell/Vesicular Stomatitis virus system, bu[t] different from that found, when human inter[-]feron was assayed in Vero or Human ski[n] fibroblasts using Herpesvirus Hominis a[s] challenge It was suggested, that the slope o[f] the regression line depends on the challeng[e] virus, while the interferon and the cell syster[m] used have only minor influence

The present work was done to extend thes[e] observations to other cell/virus systems an[d] thereby confirm the previously indicated d[...]

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minating influence of the challenge virus on the slope of the regression line

MATERIALS AND METHODS

Cells Human embryo lung cells were prepared from two different embryos of gestational age of 8 and 16 weeks (HEL_8 and HEL_{16}) Lungs were removed, cut into pieces of approx 1 mm³ size and trypsine versene treated at 37° C under gentle stirring with a magnetic bar for 45 minutes The trypsine-versene was then removed by low speed centrifugation and the cells suspended in growth medium equal volumes of medium 199 (Grand Island Biological Co, San Francisco Calif, GIBCO) and Eagle s medium with Hanks salts (GIBCO) supplemented with 10 per cent calf serum, 0 022 per cent $NaHCO_3$ and antibiotics, approx. 200 ml of medium per ml of packed cells The cells were maintained in the same medium but with 5 or 2 per cent calf serum and 0 088 or 0 132 per cent $NaHCO_3$ Throughout this study passage level 5 9 was used

Human embryo skin cells (HES) were prepared from the 8 weeks old embryo They were treated as described for HEL cells and passage level 5 9 was used in this study

A continuous line of human amnion cells, the U line was received from Dr *K Cantell* University of Helsinki, Finland The cells were grown in Eagle s medium supplemented with 10 per cent calf serum 0 044 per cent $NaHCO_3$ and antibiotics and maintained in the same medium but with 5 or 2 per cent calf serum and 0 132 per cent $NaHCO_3$

Human skin fibroblasts (HSF) from normal adult were received from Dr *H Bondevik*, Rikshospitalet, Oslo They were grown in medium 199 supplemented with 20 per cent calf serum 0 044 per cent $NaHCO_3$ and antibiotics and maintained in the same medium but with 10 5 or 2 per cent calf serum They were used at passage level 21 24 in this study

The vero line of African Green Monkey kidney cells was received from Dr *J C Ulstrup*, Ullevål Hospital Oslo The cells were grown in medium 199 supplemented with 5 per cent calf serum 0 044 per cent $NaHCO_3$ and antibiotics and maintained in the same medium but with 2 per cent calf serum and 0 132 per cent $NaHCO_3$

The $L\text{-}F_1$ line of mouse fibroblasts was received from Dr *S Haahr*, University of Aarhus Denmark They were grown in Eagle s medium supplemented with 10 per cent $NaHCO_3$ and antibiotics and maintained in the same medium but with 5 or 2 per cent calf serum and 0 132 per cent $NaHCO_3$

Mouse embryo fibroblasts (MEF) were prepared from whole mouse embryos by the same technique as for human embryo cells The cells were grown in Eagle's medium with 10 per cent calf serum 0 044 per cent $NaHCO_3$ and antibiotics and maintained in the same medium but with 5 or 2 per cent calf serum and 0 132 per cent $NaHCO_3$ Secondary cultures were used for all experiments in this study Mouse sarcoma cells were prepared from 3 Methylcholanthrene induced subcutaneous tumours received from Dr *A Elgjo*, Rikshospitalet Oslo They were treated as the MEF cells and passage level 3 5 was used in this study

Interferon Human interferon was kindley given to us by Dr *K Cantell*, University of Helsinki Finland It contained 20,000 units per ml and was stored at –20° C A crude preparation of mouse interferon was prepared in $L F_1$ cells Two days old $L F_1$ cells were incubated with egg grown Newcastle Disease virus (NDV) at 37° C for one hour Then the virus was removed, the cells washed once with medium and incubated with maintainance medium for 24 hours at 37° C The medium was then removed, adjusted to pH 2 with 1 N HCl and placed at +4° C for 3 days before addition of 1 N NaOH to neutral pH The crude preparation was filtered through Millipore filter and stored at –20° C It contained 3 200 units per ml when assayed by the micro method described in this study

Virus Vesicular Stomatitis virus (VSV), Indiana strain, was used as challenge virus in part of this study It was passed two times in the allantoic cavity of embryonated hens eggs and then several times in $L F_1$ cells The virus was stored at –70° C and for each experiment a fresh ampoule was thawn immediately before use

Herpes virus type 1, Tyler strain was received from Dr *P Leinikki*, University of Helsinki Finland It was passed in Vero cells and stored at 70° C after addition of 10 per cent fresh rabbit serum

Herpes virus type 1, McIntyre strain and type 2, MS strain were received from Dr *Faber Vestergaard*, University of Copenhagen Denmark Type 1 was passed two times in Vero cells and then stored at –70° C after addition of 10 per cent fresh [illegible] n Vero [illegible] e addi[illegible] storage [illegible] at 70° C

Newcastle Disease virus (NDV) was used as inducer in the production of mouse interferon It was passed three times in the allantoic cavity of embryonated hens eggs and allantoic fluids with high hemagglutination titres were pooled and stored at –20° C

Microequipment The equipment as well as washing and sterilization methods used were the same as earlier described (2)

Interferon assay The micro method for assaying human and mouse interferon employed in this study has been described in detail elsewhere (2) In short serial two-fold dilutions of interferon were prepared and transferred in 0 1 ml volumes per cup

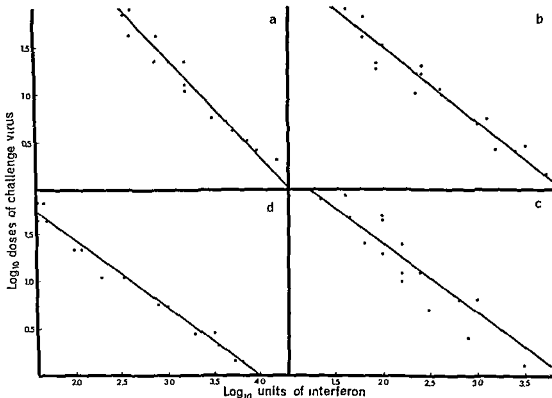


Fig 1 The regression lines for human interferon in HEL_8 cells challenged with the different viruses a. Vesicular Stomatitis virus b Herpes virus type 1, Tyler strain c Herpes virus type 1, McIntyre strain and d Herpes virus type 2, MS strain

to microtrays simultaneously seeded with 20 000 cells in 0 1 ml of medium The tray was then sealed with cello-tape and incubated over night before challenge with the chosen dose of virus in 0 025 ml of medium. A virus titration was run in parallel Microscopical reading was done after three more days of incubation at 37° C with tape sealing As a rule a regulation of pH was necessary at the second day after virus challenge This was carried out as earlier described (2)

Because of the slow metabolism of the human embryo cells a slight modification of the procedure was necessary when these cells were used After addition of interferon and cells to the trays they were covered with sterilized alu foil and incubated in the CO incubator at 37° C over night The next day the trays were challenged and sealed as usual and then incubated at 37° C for three days without further regulation of pH before microscopical reading for inhibition of viral CPE

Calculation of regression lines Regression lines illustrating the influence of challenge virus dose on the resulting titre of interferon were calculated as the least squares line according to the formula

$$Y = \overline{Y} + b\ (X - \overline{X}),\ \text{where}$$

$$b = \frac{\Sigma (X - \overline{X})\ (Y - \overline{Y})}{\Sigma\ (X - \overline{X})^2}$$

represents the slope of the calculated line X is the titre of interferon and Y is the dose of challenge virus used to obtain this interferon titre both expressed in $\log_{10}$ units Each regression line in this study is calculated on the basis of 14 20 titrations i e related values of X's and Y s

RESULTS

Dose-Response for Human Interferon Challenged with Vesicular Stomatitis Virus

Human interferon was assayed in several different human cell strains and in Vero cells by the method described in materials and methods The cells were challenged with in

TABLE 1 *The Slopes of Regression Lines for Human Interferon in Different Cell Systems Challenged with VSV*

Cell strain or line	Slope of regression line, b
HES	−1 113 ± 0 29†
U cells	−1 082 ± 0 23
HSF	−1 132 ± 0 25
HEL_8	−1 065 ± 0 11
HEL_{16}	−1 034 ± 0 13
Vero	−0 957 ± 0 18
Mean	−1 064§

† 95 per cent confidence limit
§ Statistical identity ($p > 0\ 50$, t test)

creasing doses of VSV in 0 3 or 0 5 $\log_{10}$ steps using at least 4 cups per interferon dilution per virus dilution Interferon titres were calculated by the method of Reed and Muench and corresponding values of interferon titres in $\log_{10}$ units per 0 1 ml and virus doses used in $\log_{10}$ $TCID_{50}$ units per 0 025 ml were used in the compution of regression lines for each cell strain In figure 1a the regression line for the HEL_8/VSV is used as an illustration Table 1 gives further results from this experiment The slopes of the six different regression lines are found statistically identical ($p > 0\ 50$ t-test) independent of cell system used

Dose Response for Human Interferon Challenged with Three Different Herpes Virus Strains

In order to find out if the slope of the regression line is changed when the same interferon is assayed with a different challenge virus the same experiment was repeated using three different Herpes virus strains as challenge Two strains of Herpes virus type 1 and one strain of type 2 were used Figure 1b-d illustrates the resulting regression lines for HEL_8 cells when challenged with each of the three Herpes virus strains In Table 2 the slopes of the regression lines found are given for each Herpes virus strain With in strain the slopes were statistically identical and the two type 1 strains gave identical slopes ($p > 0\ 45$, t test), while the type 2 slopes appeared to differ a little, but significantly ($p < 0\ 01$, t test) from those found with the type 1 strains All three Herpes virus strains, however, resulted in slopes significantly different from the slopes found when VSV was used as challenge ($p \ll 0\ 01$, t-test)

Dose-Response for Mouse Interferon Challenged with Vesicular Stomatitis Virus

Mouse interferon was assayed as described for human interferon in three different mouse

TABLE 2 *The Slopes of Regression Lines for Human Interferon in Different Cell Systems Challenged with Herpes Viruses*

Cell strain or line	Slope of regression line, *b* after challenge with		
	Herpes 1 Tyler strain	Herpes 1 McIntyre strain	Herpes 2, MS strain
HES	−0 702 + 0 07†	n d*	n d
HSF	−0 726 ± 0 07	−0 687 ± 0 09	−0 782 ± 0 05
HEL_8	−0 725 ± 0 11	−0 717 ± 0 15	0 744 ± 0 07
HEL_{16}	−0 702 ± 0 18	−0 755 ± 0 11	−0 770 ± 0 12
Vero	−0 680 ± 0 12	−0 707 ± 0 11	−0 740 ± 0 05
Mean	−0 705§	−0 717§	−0 759§§

† 95 per cent confidence limit
* Not done
§ Statistical identity with in strain and between the two Herpes virus type 1 strains ($p > 0\ 45$, t test)
§§ Statistical identity with in strain ($p > 0\ 50$ t test) and significant difference from the two Herpes type 1 strains ($p < 0\ 01$ t test)

TABLE 3 *Slopes of Regression Lines for Mouse Interferon in Different Cell Systems Challenged with VSV*

Cell strain or line	Slope of regression line, b
L-F$_1$	−1 116 ± 0 23†
MEF	−1 106 ± 0 105
Mouse sarcoma cells	−0 954 ± 0 071
Mean	−1 059§

† 95 per cent confidence limit
§ Statistically identical with slopes found for human interferon challenged with VSV ($p \gg 0\ 50$ t test)

cell systems using VSV as challenge in order to see, what would happen to the slope of the regression line, when a different interferon was tested with the same challenge virus The results of this experiment are given in Table 3 The resulting slopes of regression lines are found statistically identical with each other and with the slopes found when human interferon was assayed with VSV as challenge ($p \gg 0\ 50$, t test) These results are in good agreement with earlier experiments (2)

DISCUSSION

The value of a biological test depends to a large extent on the sensitivity and the reproducibility of the test. The interferon assay described in this study has been shown to be as sensitive as the commonly employed plaque reduction assay (2) The reproducibility of the test is, however, strongly influenced by smaller variations in virus challenge dose thus a correlation to a standard dose of challenge virus is necessary in order to obtain satisfactory reproducibility

A straight line relationship is found between the interferon titre obtained and the dose of challenge virus used when both are expressed in $\log_{10}$ units The challenge with the same virus resulted in identical slopes of regression lines, whether human or mouse interferon were tested and independent of the cell system used, while challenge with different viruses gave significantly different slopes of regression lines Taking advantage of these results interferon titres can easily be related to the prefixed dose of challenge virus by interpolation along the regression line Straight line dose-responses have been shown in other interferon assay methods too (12), but without comparison between different cell/virus/interferon systems

VSV is known as at least intermediately sensitive to interferon, while Herpes viruses are less sensitive (7) These results were confirmed by *Oh* (10), who reported that more Poly I C was needed to protect primary rabbit kidney cells against Herpes virus type 1 strains than against type 2 strains, while considerably less was needed to protect against VSV, indicating, that VSV is more susceptible to interferon than Herpes viruses and Herpes virus type 2 more susceptible than type 1 The resulting slopes of regression lines for the viruses employed in this study seem to reflect these reported differences in sensitivity, as higher sensitivity resulted in a steeper slope If this is true for other viruses as well, the slope might be used as a direct expression for the relative sensitivity of a certain challenge virus

The susceptibility of a virus to interferon is reported to depend on the cell system used (8) This may be explained by differences in the cell/virus interaction or in the virus inhibiting protein released by interferon treated cells (13) However, the slopes of regression lines found in the present study were the same, independent of cells and interferon as long as the same virus was used as challenge These results may indicate that the same or strongly related virus inhibiting protein is released from different cells in response to interferon treatment and that differences in virus susceptibility to interferon in different cell systems reflect differences in cell ability to produce this protein A further study on this point would be of interest and may add some knowledge to the interferon mode of action

I wish to thank Miss *Solfrid Haldorsen* for excellent technical assistance

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IMMUNOFLUORESCENCE MEASUREMENT OF CI INACTIVATOR (ALPHA 2 NEURAMINOGLYCOPROTEIN) ACTIVITY OF THE SURFACE OF HUMAN CARCINOMA CELLS

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The authors have demonstrated *in vitro* that carcinoma cells are coated with CI inactivator The presence of CI inactivator was determined by incubating the cells with fluorescein isothiocyanate conjugated rabbit anti human CI inactivator* followed by measurement of cell fluorescence on a Leitz MPV I cytophotometer The control material consisting of cells from pleural and ascites fluid of patients suffering from non malignant diseases showed statistically a significant difference from the malignant cells The possibility that the CI inactivator functions as a defence for the carcinoma cells against the humoral immune system is discussed

The first component of the human complement system is present in inactive state in serum The component consist of a macro molecule with a sedimentation constant 18–19 S which dissociates into at least three sub units, termed Clq Clr and Cls, which in a rather complicated sequence interact with each other resulting in an esterase, called CIs

Plasmin, autocatalysis (37, 38) or an antigen antibody reaction (IgG or IgM) may be responsible for CI formation from the serum pool (5, 6 35, 36)

CI and CIs is capable of hydrolysing several aminoacid esters, among others N-acetyl L-tyrosin etylester (37 38, 43) This reaction is utilized as an indicator of CIs esterase activity In the complement system activation and functional integrity of this enzyme are requirements for the subsequent two steps involving C4 and C2 (36, 37)

The human serum contains a protein, CI inactivator, which inactivates CIs and macro molecular C1 esterase This substance was first investigated by *Levy & Lepow* (39) and called Cl inhibitor,—in the following termed CI inactivator (abbrev CI IA) The inactivator is thermolabile (56° C for 30 minutes)

Pensky et al (41) achieved by chromatographic methods a purification of CI IA to separate it from trypsin and plasmin inhibitors

Ratnoff et al (44) demonstrated the inhibiting effect of CI IA, except on CI, also on plasmin kallikrein, plasma permeability factor, and Clr subcomponent *Haupt et al*

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* The anti CI inactivator is gift from Behringwerke AG Marburg an der Lahn

(23) isolated and characterized C1 IA chemically. They found that besides several amino acids it consisted of 11 per cent hexose and about 14 per cent acetyl neuraminic acid.

Neuraminidase splits the glycoside linkages in N acetyl neuraminic acid. *Bagshawe, Currie* and other workers (1, 14, 15, 16) showed that neuraminidase renders tumour cells more immunogenic. The mode of action of the neuraminidase on the tumour cells has not yet been clarified (7). The activity of neuraminidase on tumour cells has also been described by *Simmons, Ray* and others (14, 45, 47, 48) in relation to embryonic tissues and to lymphoid cells.

Hellström and others (12, 26, 30, 33) have recorded a colony inhibition technique by which they were able to study the interaction of lymphocytes and tumour cells. Furthermore they found that serum, derived from patients having progressively growing cancer, could block the immune lymphocyte induced destruction of the neoplastic cells.

Hellström and other scientists (26, 49) postulated that the blocking effect of serum inhibits the immune lymphocyte cytotoxicity and thus enhances growth of tumour, the nature of the blocking factor is still not known. *Sjögren et al.* (50) found evidence for the blocking factor consisting of antigen antibody complexes.

Hellström et al. (28) found that the blocking effect of serum from patients with cancer, — *in vitro* incubated with neoplastic cells and immune lymphocytes, disappeared when the cell suspension mentioned above was incubated with serum from patients cured by surgery of the identical type of tumour.

Using L 5178 Y lymphoma cells *in vitro* *Denham et al.* (18) found that immune lymphocytes collected from the thoracic duct showed a strong specific cytotoxic effect on the lymphoma cells. It is doubted by many authors, whether complement plays any role in the cytotoxicity of the immune-lymphocytes but *Grant et al.* (21) found a pronounced increase in the cytotoxicity of immune-lymphocytes on addition of foetal (non heat inactivated) calf serum. With complement from Wistar rats an especially high increase of cytotoxicity was achieved, whereas heat inactivated serum did not show the same effect.

We have assumed that C1 IA is the blocking factor coating the tumour cells and in this way inhibits the complement system in its cytotoxic activity.

MATERIALS AND METHODS

Anti human C1 IA-FITC from Behringwerke (stored at + 4° C) was tested for monospecificity by immunoelectrophoresis as modified by *Ouchterlony* (40) applying 10 microlitres undilated serum from healthy donor to the punched holes in the agarose gel for electrophoresis for 1½ hours (70V). 100 microlitres of anti C1 IA FITC in a dilution 1 1 with saline (0.15 M) was applied to the longitudinal basin. After a diffusion for 72 hours followed by washing for 24 hours in saline (0.15 M) the gel was stained with Coomassie brilliant blue.

Cultivation of cells. Pleural or ascites fluid freshly taken from patients with carcinomas as well as from patients with transudates were centrifugated in a Christ II KS refrigerated centrifuge 800 rev/min 0° C for 15 minutes.

The precipitate was washed twice in phosphate buffer, pH 7.4 and centrifugated as above in Eagle modified medium and cultured in monolayer in Leighton tubes, directly on slides.

In case of contamination with erytrocytes the cell culture was rinsed carefully after 24 hours growth with phosphate buffer (pH 7.4) at 37° C and fresh medium was added. After 48 hours at 37° C the culture was incubated with antiserum.

Incubation of the cells with anti C1 IA FITC. Anti human C1 IA was conjugated a.m. *Fothergill* (20) and stored at +4° C. The medium covering the culture in the Leighton tubes was carefully poured out and 1 ml of anti C1 IA FITC was placed on the slide for incubation at 37° C for 30 minutes.

After incubation the exceeding anti C1 IA FITC was removed by washing carefully 5 times in phosphate buffer (pH 7.4) at room temperature. The slides were mounted with glycerol and sealed.

Cytophotometric measurement. The equipment for microfluorometry was the Leitz MPV I system including an Ortholux microscope fitted for fluorescence microscopy with a vertical illuminator. The U.V. light source was a stabilized xenon high pressure lamp with a stabilized power supply. Incident light was obtained from a wolfram lamp through a phase contrast condenser. The barrier filter used to exclude non specific signals was a

K 530 and the excitation filters were a BG 38/3 mm and a BG 12/5 mm. The measuring diaphragm was adjusted to about 15 micron and the field diaphragm to twice this diameter The equipment included a microphotometer attachment MPV and multiplier unit 9558 AS 20 (S 20 response)

Finally the photo current from the multiplier unit was measured on a sensitive galvanometer All measurements were carried out at the range 2×10^{-9} A per scale deviation In the following one scale deviation in this range is equal to one arbitrary working unit (w u)

The PLOEM principle, meaning that the objective acts as condenser with fixed aperture was used together with MPV 1 system

A measurement was carried out in the following way From the coverslip a cell (7 to 15 micron in diameter) was selected using incident light through a phase contrast condenser The cell was centered in the measuring diaphragm After cutting out the incident light a short exposure of U V light was given watching the galvanometer To exclude fading only the maximum value was scored A background measurement was carried out following the same procedure In this way 100 consecutive cells and backgrounds were scanned on each cover slip The deviation on the background measurements was ± 2 w u on a coversl p and about ± 4 w u on the individual coverslips due to slight variations in preparation By keeping all adjustments fixed and by using the PLOEM principle together with stable U V light the measurements were reproducable

Autofluorescence was checked and found to be negative on cells treated as the scored except for the fluorescence conjugation to the antibody

Controls

A) Trypsin pre treatment of malignant cell cultures. B) Pre saturat on of malignant cell cultures with the anti C1 IA without FITC followed by washing and a new incubation with anti C1 IA with FITC

ad A Malignant human cell cultures were incubated for 5 minutes at 37° C with trypsin (41) and washed carefully with 4 times dips in phos phate buffer (pH 7 4) The cultures were then incubated with anti C1 IA FITC and measured on cytophotometer as previously mentioned

ad B Malignant human cell cultures were incubated at 37° C for 30 minutes with anti C1 IA (without FITC) washed and incubated with anti C1 IA FITC washed and measured as above

RESULTS

The monospecificity on the anti human C1 IA FITC was tested by immunoelectrophore

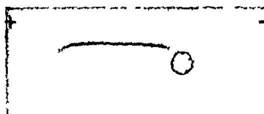


Fig 1 Demonstration of monospecificity of anti C1 IA FITC against human serum using immunoelectrophoresis as modified by *Ouchterlony* 10 microlitres human serum from healthy donors was applied to the hole punched in a 2 per cent agarose gel Electrophoresis was performed at 70 V for 2 hours 100 microlitres anti C1 IA FITC diluted 1 1 in saline (0 15 M) was placed in the longitudinal basin as antibody After 72 hours diffusion followed by 24 hours' wash in saline (0 15 M) the gel was stained with Coomassie brilliant blue

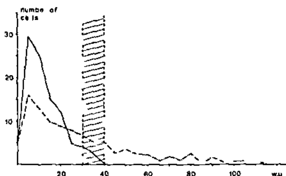


Fig 2a Anti C1 IA-FITC immunofluorescence measurements on human neoplastic cell cultures pre- saturated with anti C1 IA (without FITC) followed by incubation with anti C1 IA FITC. The full drawn curve shows the fluorescence on the pre saturated cells due to unspecificity Abscissa Photometer current in working units after background subtraction (1 w u = 2 × 10 9 Amp) Ordinate Number of scored cells

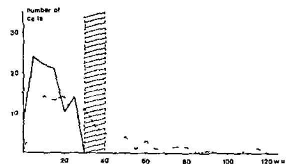


Fig 2b Anti C1 IA FITC immunofluorescence measurements on human neoplastic cell cultures treated with trypsin before incubation with anti C1 IA-FITC The full drawn curve shows the trypsin treated cells Abscissa and ordinate as in Fig 2a

Department of Sundby Hospital Copenhagen

Fortyfour patients suffering from neoplastic diseases of types and localizations described in Table 1 and eight control patients with non-neoplastic diseases constitute the basis for the assay In Fig 3 the average distribution regarding the malignant benign cells is demonstrated It is emphasized that no single score of the benign cells exceeds the threshold value for malignancy (Fig 3 a and 3 b)

The calculation basis and reliability limits for the 52 assayed patients is indicated in Table 2 The control material demonstrated

TABLE 1 *The Distribution and Type of the Assayed Carcinomas*

Cystadenocarcinoma papilliferum ovarii	7 patients
Carcinoma solidum ovarii	2 „
Adenocarcinoma papilliferum ovarii	7 „
Carcinoma solidum pars adenomatosum mammae	8 „
Carcinoma solidum scirrosum mammae	12 „
Adenocarcinoma mammae	2 „
Adenocarcinoma cervicis uteri	1 „
Carcinoma solidum renis (hypernephroma)	1 „
Adenocarcinoma colloides appendicis	1 „
Carcinomatosis peritonei, ons pro	1 „
Adenocarcinoma ovarii	1 „
Teratoma malignum ovarii	1

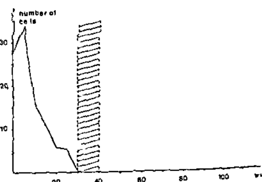


Fig 3a Anti C1 IA-FITC immunofluorescence measurements on human cells from 8 patients suffering from non malignant diseases Abscissa and ordinate as in Fig 2a

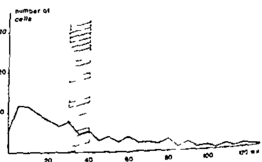


Fig 3b Anti C1 IA-FITC immunofluorescence measurements on human cells from 44 patients suffering from neoplastic diseases Abscissa and ordinate as in Fig 2a.

reliability for at least 63 per cent of the scores, the malignant material at least 92 per cent of the scores The significant difference p is < 0.002 (Fisher's exact test)

TABLE 2 *Distribution of Benign Malignant Human Cell Cultures Using Cytophotometric Measurements on Anti CI IA FITC and Statistical Calculations on the Confidence Limits*

	Scored values ≤ 30 w u		Scored values > 30 w u	
	Number of patients	Statistical confidence	Number of patients	Statistical confidence
Benign	8	63–100 %	0	0– 37 %
Malignant	0	0– 8 %	44	92 100 %

The d fference between ben gn and malignant human cell cultures was significant at a 0 002 level based on Fisher s exact test

DISCUSSION

Several authors have demonstrated, that tumours produce specific antigens, absent in normal tissue (2, 19, 22, 34, 42) Using colony inhibition technic, *Hellstrom* and others have found immune lymphocytes able to repress or inhibit tumour cell colony growing in mono layer cultures (27)

A number of papers (3 4, 17, 27, 30 32, 49) show that factors in autologous serum are able to block the colony inhibition

Hellström put forward two alternative hypothesis The first theory is based upon the fact that the amount of immune lymphocytes is too small to destruct the neoplastic cells The other points at the possibility that the cytotoxic activity of lymphocytes *in vitro* is inhibited by factors present in culture systems, working on lymphocytes or rather on target cells These factors are termed enhancing antibodies or tolerogenic antigens (31)

Other authors, among these *Denham et al* (18) and *Grant et al* (21) have given evi dence that the cytotoxic reaction by immune lymphocytes consists of immunoglobulin and complement like factors *Grant et al* has furthermore shown that in a system consisting of immune lymphocytes in suspension sep arated from the target cells with a millipore filter with a pore diameter of 0 45 micron the destruction of target cells was increased when adding complement to heat inactivated suspension or when the lymphocyte target cell rate was 100 1 Other authors have shown that in a complement inactivated system the immune lymphocytes should be in close con nection with the target cells for efficient cytolysis (10, 11, 46)

In order to find a factor, which could unite the theories of a possible blocking effect of complement activity on cells with the hypo thesis of *Hellström* and other scientists re garding the blocking factors we decided to examine a possible coating of neoplasm cells with CI inactivator With a coating with CI inactivator it would be difficult or impossible for the humoral immune system to attack the cells

This working hypothesis opposes in a way the problems concerning the blocking factors The hypethesis may explain *Hellström*s theory regarding enhancing antibodies or tolerogenic antigens on target cells

Another reason for the relation between CI inactivator and neoplasm cells is based on recent observations made by *Currie* and others (1, 14, 15, 16) demonstrating that neura minidase from *Vibrio cholerae* will enhance the immunogenecity of neoplasm cells Neura minidase decomposes neuramine compounds of which CI inactivator (N acetyl neuramino glycoprotein) consists

Von Zeipel (51) has recently reported occurrence of CI esterase, together with two other esterases in serum free medium where cells of various origin among those cervix cancer were cultured Furthermore he found an inhibition phenomenon, predominantly in cells extracts The phenomenon was temper ature dependent (37° C), affecting all three types of esterases These results were repro ducable after several subculturations In rela tion to *von Zeipel*'s findings in subcultures,

we found CI IA attached to the tumour cells through several trypsinized subcultures in Eagle medium containing inactivated foetal calf serum

Cytophotometry has proved a valuable tool in immunology In this way weak reactions can be detected in a reproductable system In consecutive pilot assays we used ordinary fluorescence microscopy To get more objective informations and to correlate the individual results, we decided to utilize microfluorometry In this way the results may be presented in arbitrary working units and choosing adequate references the results can be quantitated

It was of interest to examine whether the FITC conjugated anti C1 IA reacted specifically with the assumed CI IA on the cells The cells were pre saturated with unconjugated anti C1 IA (Fig 2 a) The results render it probable that a specific binding took place

The values scored in the area 0–40 w u on the pre saturated cells are explained as unspecific binding

It is shown (44) that trypsin may decompose, among other proteins, CI IA We have therefore pretreated our malignant human cell cultures with trypsin (Fig 2 b) resulting in an elimination of the assumed specific binding of CI IA on the malignant cells The remaining fluorescence is explained as above

Results of the preceding experiments intimate the zone between benign and malignant values at 30 and 40 w u (hatched area) This area serves as guidance only since the statistical confidence is outside the border lines

Measurements on the patients described in methods and materials were carried out As the results were based on cells from pleura and ascites fluid it was difficult to obtain bigger quantities of controls The mathematical calculations over the confidence to the groups benign malignant demonstrate low values for the benign cells (Table 2) Still the interest was concentrated about a clear separation, which based on Fisher's exact test proved a significant difference

Recalling that complement together with antibodies (IgG, IgM) comprises the cytotoxic principle in the humoral immune defence, it is possible that the presence of the CI IA on the malignant cells may explain, why this defence system does not play any part in malignant diseases In this way the neoplasm cells may be protected against the humoral immunesystem Furthermore it could be explained why *Hellström* and others were not able to demonstrate any cytotoxic effect of autologous serum on neoplasm cultures

The demonstration by *Hellstrom* and *Sjogren* of the blocking factor in serum from patients suffering from cancer—against the cellular immune system makes it evident that other blocking systems are protecting the cancer cells

Even if the assays in the first place have been aimed at the basic theoretic aspects we must not omit to point out that the method could be utilized in the diagnosis of cancer As an example suggesting a practical application of the method for demonstration of CI IA on malignant cells we may mention a patient with an assumed benign course for her ascites, which showed CI IA activity of a certain number of the ascites cells On succeeding laparoscopy it was proved that the patient had peritoneal carcinosis

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COMPLEMENT FACTORS AND THE GROWTH OF EHRLICH'S CARCINOMA

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The subcutaneous growth of Ehrlich's carcinoma and the acute inflammatory reactions against it were investigated in mice treated with the following complement depleting agents heat aggregated human gamma globulin (HAGG), zymosan, anti C1q and anti C3 In normal mice deposits of mouse C1q, C3 and IgG in and around small vessels adjacent to the tumour were paralleled by a marked inflammatory response with oedema and accumulation of leucocytes In contrast, mice treated with zymosan showed reduced serum C3 levels, lack of C3 binding to walls of vessels and lack of leucocyte infiltration Serum C1q levels were normal in zymosan treated mice Moreover, HAGG and anti C1q reduced serum C1q significantly without affecting the inflammatory reactions These findings emphasize the important role of C3 as a leucotactic factor Mice depleted of C3 by zymosan showed decreased tumour size and tumour cell infiltration in addition to lack of inflammatory response It is suggested that the availability of C3 is a determining factor in the establishment and early growth of this tumour

The role of complement factors in immunologic (17), as well as non immunologic (19) inflammation is well established Depletion of complement *in vivo* reduces the extent of both reactions Subcutaneous transplants of the Ehrlich carcinoma in normal mice elicit an acute inflammatory reaction (6), which can be reduced by treatment with agents known to inhibit complement activity (7) Moreover, inhibition of inflammatory reactions was paralleled by a reduction in tumour size, suggesting that complement plays a part in tumour growth

In the present study individual serum complement components were measured following *in vivo* treatment of mice with complement depleting agents during growth of Ehrlich's carcinoma The relationship of serum complement components to the presence of tissue bound complement was investigated and compared to tumour growth and the inflammatory reactions against it

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MATERIAL AND METHODS

Mice Male mice of the closed colony (CC) kept at this Institute were used (7) All mice were 6-8 weeks old, weighing between 20-25 g

Tumour The Ehrlich carcinoma (14) used was kept in CC mice by serial intraperitoneal transplantation One tenth ml (7 8 x 10^6 tumour cells) of tumour ascites was given subcutaneously on the back

Tissues The skin of the back with subcutaneous tumours was removed The size of the tumours and the size of the tumour plus its surrounding oedema were measured macroscopically (sum of two greatest diameters at right angles) Erythrocytes present in the injected tumour ascites tend to gather at the periphery of the oedema around the tumour (7) They were therefore used as a marker for the outer border of the reaction site The skin with the tu-



Fig 1 Double diffusion gel precipitation between rabbit antiserum to mouse Clq (top) and fresh mouse serum (bottom right) Fresh mouse serum heated for 5 min at 56° C (bottom left) is negative The sera are tested undiluted

mours were then bisected One half was fixed in 4 per cent buffered formalin and paraffin embedded Sections for histology were stained with haematoxylin and eosin The other portion of tissue was frozen and stored at −20° C

Sera Whole blood taken either from the retro-orbital plexus or from the axillary vessels was allowed to clot in an ice-water bath for 30 min The serum was separated by centrifugation at 1000 g for 10 min at 4° C and stored at −20° C Serum samples were tested within 3 days of collection

Heat-aggregated gamma globulin (HAGG) Human IgG was purchased from AB Kabi Stockholm It was aggregated by heating a 10 per cent (w/v) solution in phosphate buffered saline pH 7 2 (PBS) at 63° C for 10 min The HAGG was made up to a 1 per cent solution for injection

Zymosan Zymosan (Sigma Chemical Co St Louis Miss) was boiled for 30 min in 20 volumes of PBS The pellet obtained by centrifugation was resuspended in PBS to a suspension containing 10 mg/ml

Immunoelectrophoresis was carried out with LKB equipment (Gelman Instrument Co Ann Arbor Mich) for 1 hour at 250 V

Double gel immunodiffusion was performed in 0 85 per cent agarose (w/v) as described in a previous paper (15)

Sera Normal sera from rabbits and CC mice were obtained from the animal quarters Rabbit antiserum to mouse IgG was purchased from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service Amsterdam Anti mouse Clq was produced by injection into rabbits of Latex particles (Bacto-Latex 0 81μ Difco Laboratories Detroit, Mich) agglutinated by normal mouse serum in the presence of 0 01 M Na EDTA The procedure was similar to that previously described in detail for the production of rabbit anti human Clq (15) Double gel diffusion of this antiserum against fresh normal mouse serum showed a single precipitin line (Fig 1) Heating of mouse serum at 56° C for 5 min completely abolished the reaction Rabbit anti mouse β1C/β1A (C3) was produced as described by *Mardiney* & *Muller Eberhard* (10) Fig 2 shows the immunoelectrophoresis pattern of this antiserum against fresh normal mouse serum The activity was directed mainly against β1C but a weak line appeared in the β1A region as well In double gel diffusion tests with the same sera or mouse plasma in 0 01 M Na EDTA only one precipitin line was seen

IgG fractions of the sera were prepared as de



Fig 2 Immunoelectrophoresis of fresh mouse serum (well) developed with rabbit antiserum to mouse C3 (upper trough) and rabbit anti whole mouse serum (lower trough) The anti-C3 serum detects mainly β1C, but a weak line is seen in the β1A region as well The sera are tested undiluted

scribed previously (16) All sera were heat inactivated at 56° C for 30 min Sodium azide, 0 1 per cent, was added as a preservative

Immunofluorescence methods IgG fractions of antisera were labelled with FITC to a molar ratio of 1 5 to 4 Free dye was removed by gel filtration (Sephadex G 25) and nonspecific staining reduced by absorption with rat liver acetone powder (11)

For direct immunofluorescence cryostat section (4μ) of frozen samples of skin with tumour tissue were air dried and washed for 2 hours at 4° C in PBS with continuous stirring The sections were treated with conjugate for 30 min at 37° C washed with PBS for 30 min and mounted in a mixture of glycerol and PBS (1 in 1) The cover glasses were sealed with nail varnish Stained sections were examined by a Leitz Orthoplan microscope using a HBO 200 W UV light source Kodak high speed Ektachrome film was used for photomicrography

Complement depletion in vivo Complement depletion was carried out by repeated intravenous injections in 0 1 ml volumes of 1 mg HAGG, 1 mg Zymosan and 0 1 ml of whole rabbit anti mouse C1q or C3 at the time intervals indicated in Fig 5 Control mice received equivalent volumes of normal rabbit serum or PBS Prior to injection all reagents were filtered through a Millipore filter, pore size 0 45μ

Analysis of serum complement and IgG levels The complement components C1q and C3 as well as IgG were quantitated by single radial immunodiffusion (5, 9) as modified by *Brandtzaeg et al* (1) In brief 5μl volumes of sera to be tested were added to each well in plates containing a mixture of equal volumes (2 0 ml) of appropriately diluted IgG fractions of antisera and 3 per cent agarose The precipitation patterns were developed in a moist chamber at room temperature for 48 hours Serial dilutions of a serum pool obtained from 20 normal mice were placed in 5 wells on each slide This reference pool was used as a standard for all assays, the undiluted pool being designated as 100 per cent The levels of C1q and C3 in the individual sera of the pool differed from this value by ± 15 per cent

White blood cell count Total white cell counts were done in a Burker haematocytometer on blood samples obtained before and after complement depletion procedures in some of the mice in each group

Experimental Procedures

Complement depleting reagents were given 1 hour prior to tumour transplantation and at regular intervals afterwards as indicated by arrows in Fig 5 Serum samples for quantitation of complement and IgG were taken from the retroorbital plexus at regular time intervals The mice were finally bled from the axillary vessels under ether anaesthesia 48 hours after transplantation The skin with the tumours were removed and examined as described above

RESULTS

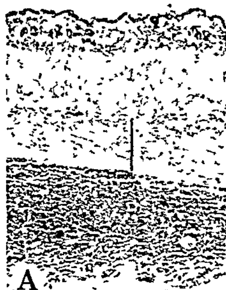
Macroscopical examination The mean diameters of the tumours and of the tumours plus its surrounding oedema are shown in Table 1 Compared to controls treated with PBS, injections of zymosan caused a marked decrease in the size of the tumours as well as in the surrounding odema Treatment with HAGG, anti C1q or anti C3 did not significantly affect tumour growth

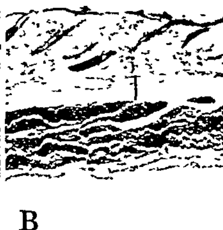
Microscopical examination The tumour transplants in the controls (Fig 3A C) showed early infiltrative growth Most tu

TABLE 1 *Measurements of Tumour Transplants§ at 48 Hours in Mice Given Various Complement Depleting Treatments Compared to Controls*

Treatment§ i v	No of mice	Mean sum of 2 tumour§ diameters ± S D	Mean sum of 2 diameters of reaction site§ ± S D
Zymosan	12	6 0 ± 1 3*	8 0 ± 2 3†
HAGG	5	8 4 ± 1 8	14 2 ± 2 8
Anti C1q	5	8 0 ± 2 0	14 8 ± 3 3
Anti C3	5	7 3 ± 3 3	12 9 ± 3 1
Rabbit serum	5	7 2 ± 1 3	12 8 ± 1 6
PBS	5	9 1 ± 1 6*	15 5 ± 2 7†

§ see text
* difference 0 01 > P > 0 001
† difference 0 001 > P





C



mours showed massive necrosis at the center of the transplant There was oedema and *marked interstitial infiltration of* leucocytes at the tumour periphery and in the necrotic centre, as well as around small vessels adja cent of the panniculus carnosus

In contrast, the edge of the tumour trans plants in the zymosan treated mice (Fig 3B D) was well demarcated from the surround ing tissue Oedema and host cell infiltration were lacking and there were no perivascular cell infiltrates Treatment with anti C3 also gave a slight decrease in host cell infiltra tion while HAGG and anti C1q had no ef fect

Tissue bound complement Direct fluorescent studies on tissue from control mice treated with PBS or normal rabbit serum re vealed the presence of bright granular depo sits of mouse C1q and C3 mainly in vessel walls, but also in the perivascular areas (Fig 4A) Mouse IgG was found with a distri bution identical with that of the complement *factors as shown by serial frozen sections in* which the same vessels could be assessed for all three reactants

On the other hand when tissues from zymosan treated mice were examined by the fluorescent antibody technique, at little IgG and C1q were found in a linear distribution along the vessel walls but no C3 was present (Fig 4B) Tissues from mice treated with anti C3 showed small deposits of C3 in the vessel walls, while C1q and IgG deposits were normal Treatment with HAGG and anti C1q reduced tissue deposition of C1q while C3 and IgG deposits were not affected

Serum complement The serum levels of C1q and C3 in the various groups are given in Fig 5 The arrows indicate the time of tumour transplantation and times of injection of complement depleting agents Neither C1q nor C3 levels changed in control mice re ceiving tumour transplants and PBS or nor mal rabbit serum

In contrast, C1q levels dropped almost immediately by 50 60 per cent following intravenous treatment with HAGG or anti C1q At the end of the 48 hour period C1q levels in the HAGG treated group were still below normal values, whereas there was a return to normal in mice treated with anti C1q In the groups treated with zymosan and anti C3 C1q values were reduced by 15 25 per cent

Treatment with zymosan caused a 50 per cent drop in C3 levels whereas treatment with HAGG and anti C3 caused a 20 per cent drop in C3 levels with return to normal values by 48 hours Treatment with anti C1q did not affect C3 levels

The serum levels of IgG were estimated quantitatively in the same way They remain ed within normal range in all groups

Peripheral white blood cells There was no reduction in white blood cell counts fol lowing treatment of mice with complement depleting agents On the contrary, the treat ments were associated with a slight increase in the peripheral white blood cell count in some mice

Fig 3 Microphotographs of subcutaneous trans*plants of Ehrlich s carcinoma at 48 hours in normal* (A C) and zymosan treated (B D) mice

A Tumour transplant with marked oedema and leucocyte infiltration in part cular below the pan niculus carnosus (|) (H E x 35)

B Tumour transplant with lack of leucocyte in filtrates Note difference in oedema between the edge of the tumour and the panniculus carnosus (|) in A and B (H E x 35)

C Tumour transplant showing infiltrative tumour growth in the panniculus carnosus central necrosis and infiltration of leucocytes in and around walls of vessels at the tumour periphery (H E x 150)

D Tumour transplant showing well demarcated tumour growth and lack of interstitial and peri vascular leucocyte infiltrates (H E x 150)

DISCUSSION

The data presented here confirmed the pre vious findings that the Ehrlich carcinoma induces an acute inflammatory reaction in normal mice (6) Moreover, it was found that a correlation existed between fixation of complement in and around vessels adja cent to the tumours, and oedema and accu



Fig 4 Fluorescent microphotographs from edge of subcutaneous transplant of Ehrlich's carcinoma at 48 hours from normal (A and zymosan treated B) mouse The sections are stained for mouse complement (C3 (× 750

A Mouse C3 is present in a granular pattern mainly in the wall of a small vessel Small deposits are seen in the interstitial tissue Mouse C1q and IgG was found in an identical distribution

B No mouse C3 is seen

mulation of leucocytes The evidence for this is based on the finding that in mice where serum C3 levels were reduced by injection of zymosan C3 was not bound to tissue lesions and vessel damage with oedema and infiltration of leucocytes did not occur

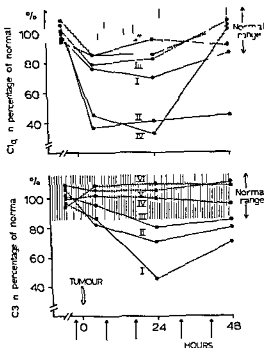


Fig 5 Mean serum levels of complement factors C1q and C3 in mice with Ehrlich's carcinoma repeatedly injected with complement depleting and control reagents (arrows) Zymosan I HAGG II anti C3 III anti C1q IV normal rabbit serum V and PBS VI The shaded areas represent the range in normal values

The latter phenomenon was probably due to lack of local leucotactic factors as a reducing effect of zymosan on the number of circulating leucocytes could be excluded In contrast in control mice the presence of C3 in the tissue was paralleled by vascular damage causing oedema as well as marked infiltration of leucocytes

The data also showed a correlation in mice treated with zymosan between serum C3 levels and the local binding of C3 in the tissues As serum as well as tissue bound C1q were only slightly affected by treatment with zymosan the lack of oedema and leucocyte infiltration in these mice indicates the important role of C3 as a mediator of the acute inflammatory reaction to the tumour cells This was in agreement with recent findings that the mid portion of the complement sequence i e a fragment of C3 (C3a) as well as $\overline{C567}$ and C5a and not the first complement components are important mediators of leucotaxis and vessel damage in acute immunologic and non immunologic inflammation (4 18) The *in vitro* combination of zymosan with C3 has been used for the inactivation of C3 in serum as well as for the production for specific antisera against C3 The results of the present experiment showed that a correlation exists between the activity of this agent *in vitro* and *in vivo*, and that the marked anti inflammatory activity of zymosan most probably was mediated through its capacity to inactivate C3 On the other hand anti C3 was without marked effect on serum and tissue bound C3 as well as on inflammatory reactions This was in contrast to previous studies in rats where *in vivo* treatment with anti C3 was found to have a marked anti complementary as well as anti inflammatory effect (19)

Treatment of mice with HAGG reduced serum levels of C1q significantly, while C3 levels showed a slight drop only This is in line with the strong *in vitro* activity of C1q for HAGG (15) Moreover the occurrence of marked inflammatory lesions in mice treated with HAGG or anti C1q where serum levels and tissue bound C1q were reduced indicated that later complement components may be activated even in the absence of early components If availability and fixation of C1q is reflected by its serum levels these data suggest that this complement component was not involved in the chemotaxis of leucocytes However, a reduction in C1q levels does not necessarily exclude a certain activity on the part of this complement component

Decreased tumour size with lack of inflammatory exudate was demonstrated in mice with low serum levels of C3 This observation suggests that the early infiltrative growth of the tumour depends at least in part on the availability of this complement component This is in keeping with the previous observation that zymosan suppresses the subcutaneous growth of the Ehrlich car

cinoma (7) It has also been previously shown, as is demonstrated in the present work, that infiltrative growth of this tumour is associated with the presence of an acute inflammatory reaction In its absence such growth is expansive rather than infiltrative, as for example in mice treated with the anti-inflammatory agent butazolidine (8), in mice treated with polyethylene glycol (6) and in the presence of a previous intraperitoneal transplant of the same tumour (7)

The importance of C3 in imune reactions involving serum antibody has been well established by several workers (see 3) Recent work indicates that C3 may play a part in delayed type hypersensitivity reactions as well (12, 19) On the other hand, absence of haemolytic complement and deficiency in a component of the C3 group in B 10 D2 *old* mice did not affect rejection of skin allografts in these mice (2) Previous studies have suggested the existence of humoral (14) as well as cellular (13) immune reactions to Ehrlich's carcinoma This is in keeping with the allograft nature of this tumour The results of the present experiment therefore suggest that the allograft reaction involving C3, rather than destroying the tumour graft is beneficial, being associated with infiltrative growth

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BRIEF REPORTS

STABILITY OF HUMAN LEUKOCYTE INTERFERON TOWARDS HEAT

K E Mogensen and K Cantell

Early reports on the stability of human interferon are conflicting (1) More recently (2) human interferon has come to be regarded as a labile substance The stability of an interferon preparation is an important factor in the assessment of its suitability for clinical use Human leukocyte interferon has been used for clinical trials (3, 4) and here we report on the stability towards heat of a crude concentrated form (C-IF) and a partially purified form (P IF)

Interferon was prepared from human leukocytes (5) with Sendai virus in the presence of human serum (6) Interferon was assayed by the reduction of vesicular stomatitis virus plaques on U (human amnion) cells (5) and all titres were expressed in international reference units/ml (7) Interferon samples in rubber stoppered glass tubes were incubated in a Memmert oven, with a temperature fluctuation of ± 0 5° C Tubes were removed at intervals, plunged into ice salt mixtures and later stored at +4° C until assay C IF was concentrated some 30 fold (6) to give 10^6 units/ml and 55 mg/ml of protein (8) in phosphate buffered saline pH 7 3 (PBS)

In contrast to *Marshall et al* (9), who found a halflife for human interferon of 200 mins at 37° C, the decay of C IF was very slow below 50° C A loss of one log unit potency required approximately 6 weeks at 37 C and 3 weeks at 45° C It was not poss[illegible]bl[illegible] [illegible]o follow the kinetics of decay with such small [illegible] Above 50° C the rate of decay increas[illegible] [illegible]ith increasing temperature until, at 90° C, it w[illegible] [illegible] rapid to follow

Interferon w[illegible] [illegible]artially purified (P IF) by the selective precip[illegible]tion of proteins at different pHs from an ethanolic solution (10) to a titre of 10^5 units/ml and 1 mg/ml of protein (8) in PBS This material was found to be more stable than C IF, e g at 56° C and pH 7 2 C IF dropped one log unit of activity in 12 hours whereas P IF still retained all activity after 3 days The stability of the P IF preparation was found to be unaffected by pH 2 0 and 9 5

While C-IF tends to gel on heating, solutions of

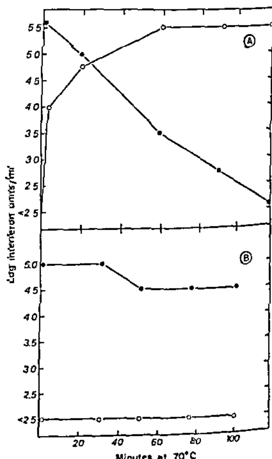


Fig 1 The distribution of interferon activity between supernatant and sediment after heating at [illegible]

A) crude concentrated interferon (C IF), B) partially purified interferon (P IF) ●—●, supernatant interferon ○—○, pellet interferon

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TABLE 1 *The Effect of Boiling and Autoclaving on Partially Purified Human Leuko...* (P IF)

Treatment	Gu HCl*	Interferon units/ml		Per cent
		Initial	Final	
Boiling†	None	200,000	1,100	0 5
Boiling	After	200,000	1,100	0 5
Boiling	Present	200,000	60 000	33 3
Autoclaving§	None	200,000	200	0 1
Autoclaving	After	200,000	200	0 1
Autoclaving	Present	200,000	2,000	1 0

* Gu HCl at a final concentration of 4M was present during the treatment (Present) or was added afterwards (After) *NaOH* was added to maintain the pH at 7 2

† Boiling was by gentle refluxing at pH 7 2 for 60 minutes

§ Autoclaving was at 120° C for 35 minutes at pH 7 2

P IF remain clear and show no visible evidence of protein denaturation It was felt that aggregation may have been responsible for the apparent decline in C IF activity Although interferon does not normally sediment upon high speed centrifugation it was found after heating that C IF activity had transferred from the supernatant to the high speed pellet (Fig 1A) Guanidine hydrochloride (Gu HCl) which was used to dissolve the pellets, does not have any irreversible effect on interferon at concentrations up to 8 M Fig 1B shows the results of similarly treating a sample of P IF No pellet was visible and no activity sedimented We suggest that interferon was masked by the heat induced aggregation of proteins in the crude preparation and that this may lead to prejudiced estimates of stability

Table 1 shows the effects of boiling and autoclaving on P IF No extra activity can be recovered by treating with Gu HCl after either boiling or autoclaving It is however clear that the presence of Gu HCl during such treatment affords some protection to interferon *Robinson* & *Jencks* (11) have suggested that compounds such as Gu HCl can interact directly with peptide bonds and that such interactions may stabilize exposed bonds

We have found that human leukocyte interfero is quite stable but that activity can be masked b non specific effects due to impurities Contrary other experience (2), we have not found that pu fication lowers the stability or that any stabili are necessary for storage

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INHIBITION OF BACTERIAL HAEMOLYSIS ON BLOOD AGAR MEDIUM BY OXALATE OR CITRATE USED AS ANTICOAGULANTS

Knud Børge Pedersen

Sodium or potassium oxalate and sodium citrate are commonly used as anticoagulants (2) Usual concentrations of the oxalate and citrate sodium salt are 0 1 0 2 and 0 3 0 6 g/100 ml blood, respectively The influence of various concentrations of these anticoagulants on the surface growth of *Moraxella bovis* (the 9N and 9SC cell lines in ref 1), *Haemophilus parahaemolyticus*, *Escherichia coli*, *Staphylococcus aureus* (α and β haemolytic strains), *Streptococcus zooepidemicus* and *Corynebacterium pyogenes* was examined on bovine blood agar medium The results were compared with the growth on the same medium with defibrinated blood

The medium employed was tryptose blood agar base (Difco) with 5 per cent bovine blood stabilized with sodium oxalate (0 1–2 0 g $C_2Na_2O_4$/100 ml blood) or sodium citrate (0 2–4 0 g $C_6H_5Na_3O_7$ 2H_2O/100 ml blood) In each experiment stabilized and defibrinated blood originated from the same animal The plates were incubated aerobically in a dry atmosphere at 37° C Readings were performed after 6, 12, 24 and 48 hours incubation

Inhibition of haemolysis by sodium oxalate was achieved in *M bovis*, *H parahaemolyticus* and *E coli* The haemolytic ability was not influenced by oxalate in *Staph aureus*, *S zooepidemicus* and *C pyogenes* In *M bovis* initial inhibition of haemolysis was observed on plates which contained blood stabilized with 0 4 0 5 g sodium oxalate/100 ml blood Complete inhibition of haemolysis was observed with 0 6 0 7 g/100 ml In *H parahaemolyticus* partial to complete inhibition of haemolysis was found at 0 4 0 6 g 100 ml blood The initial reduction in the haemolysis of *E coli* was observed from 0 6 g/100 ml and complete inhibition of haemolysis from 0 8 g/100 ml blood Oxalate did not cause any definite alterations of colony morphology

Stabilization of blood with sodium citrate caused inhibition of the haemolytic ability in *M bovis* and *H parahaemolyticus*, whereas the other examined bacteria did not show any reduction in haemolysis. In *M bovis* initial inhibition of haemolysis was observed on plates with blood which contained 2 5 g/100 ml blood, while 4 0 g/100 ml caused a very strong inhibition of haemolysis Cultures of *H parahaemolyticus* showed a reduced haemolysis with 2 5 g/100 ml but a complete inhibition of haemolysis was observed with 3 0 g/100 ml blood A definite influence on colony morphology was not observed with media containing citrate stabilized blood

The present investigation showed that blood stabilized with an excess of sodium oxalate or sodium citrate caused an inhibitory effect on the haemolytic ability of certain bacteria as detected in surface cultures on bovine blood agar medium. No distinct influence on other surface characteristics in culture was observed With oxalate about twice the normally recommended dose caused inhibition of haemolysis, whereas higher doses of citrate were required Since haemolysis is an important criterion in the initial phases of the identification of bacteria it is essential to avoid any excess of oxalate and citrate for the stabilization of blood for bacteriological use

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The H parahaemolyticus strain was kindly supplied by Dr R Nielsen, State Veterinary Serum laboratory, Copenhagen and the E coli, Staph aureus, S zooepidemicus and C pyogenes strains by Dr J I Larsen, Institute of Microbiology and Hygiene, Royal Veterinary and Agricultural University, Copenhagen

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A STUDY ON *IN VITRO* ISOLATION OF FELINE PANLEUKOPAENIA VIRUS

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Virus isolation was performed from faeces of one cat with symptoms of panleukopaenia and from autopsy material from six out of seven cats with macroscopical lesions of panleukopaenia In a few cases a cytopathic effect was observed in feline kidney cell cultures The virus produced intranuclear inclusion bodies The inclusion bodies were detectable in stained preparations Both the cytopathic effect and the intranuclear inclusion bodies occurred irregulary and was of a transient nature Inoculation experiments were made on cats using culture material Fulminating symptoms of panleukopaenia developed in five out of eight kittens after intraperitoneal inoculation

According to *Rohrer* (1968) feline panleukopaenia also called feline infectious enteritis feline agranulocytosis and feline distemper, has a wide geographical distribution The disease has been described in domestic cats in various parts of the world (U S A , Europe Australia USSR and India), using clinical and pathological examination as the main basis for diagnosis

Any member of the family felidae is susceptible to the virus (*Goss* 1948) Experimental transmission of feline panleukopaenia to mink in the family mustelidae has been described (*MacPherson* 1956) Immunological relationship between feline panleukopaenia virus and *mink enteritis virus has been found* [illegible] in vaccination experiments by *Wills* (1952) and *Wills & Belcher* (1956) and *in vitro* by *Johnson* (1967) who demonstrated the similarity of feline panleukopaenia and mink enteritis virus in cell cultures In agreement with these findings feline panleukopaenia virus and mink enteritis virus today are regarded synonyms for the same virus (*Andrewes & Pereira* 1972) which by the International Committee on Nomenclature of Viruses (*Wildy* 1971) has been classified as a possible member of the parvovirus group Virus of this group are small ether resistant, heat stable single stranded DNA viruses which multiply in the nucleus

The viral aetiology of feline panleukopaenia has been accepted in theory for 40 years (*Verge & Christoforoni* 1928), only recently has it been possible to examine the virus in an *in vitro* system In 1964 the virus was iso[illegible]

(*Johnson* 1965, *King & Croghan* 1965)

No cytopathic effect was observed in feline kidney cell cultures inoculated with panleukopaenia material *Johnson* (1964) detected the occurrence of intranuclear inclusion bodies in stained preparations of inoculated cell cultures By studying the intranuclear inclusion in cell cultures (*Johnson* 1965) observed

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the inclusion to range through eosinophilic and basophilic stages with margination of chromatin and nuclear collapse corresponding to the stage of the intranuclear inclusion bodies observed by histological examination of the intestinal mucosa in natural cases of panleukopaenia described by *Lucas & Riser* (*1945*)

In transmission experiments with inoculation of tissue culture fluid containing leopard virus, a syndrome was produced which was indistinguishable from that produced in a group of kittens inoculated with feline panleukopaenia infected tissue, and immunity to cross challenge was established (*Johnson* 1965)

The clinical pathology, gross pathology and histopathology of feline panleukopaenia have been described by *Lawrence & Syverton* (1938), *Hammon & Enders* (1939), *Kikuth et al* (1940) and *Lucas & Riser* (1945)

Based on clinical and pathological examinations, panleukopaenia has been diagnosed in Denmark for many years (*Momberg Jørgensen 1938*) The present report describes the isolation of panleukopaenia virus in cell cultures from Danish cats and also the transmission of the virus to kittens

MATERIALS AND METHODS

The virus isolations were obtained from the following materials

1) A one-year-old male Siamese cat from the Copenhagen area It was admitted to the clinic (No 12231/69 with a history of anorexia and diarrhoea for the previous two days Examination showed the cat to be extremely depressed and weak Abdominal palpation revealed excess of gas and watery content in the intestines. There was no evidence of pain from the palpation The temperature was 37 4° C The faeces were watery Marked leukopaenia due mainly to neutropaenia was found on examination of blood The total leukocyte count was 2000/mm³ with the following distribution neutrophils 10 per cent eosinophils 9 per cent lymphocytes 80 per cent and monocytes 1 per cent The haemoglobin value was 16 9 g/100 ml and haematocrit 49 per cent The leukocyte picture indicated feline panleukopaenia Conventional treatment with fluid and antibiotics was instituted The condition improved and the cat recovered within a week The leukocyte count increased to 26 000/mm³ in four days. Swabs were taken from the rectum for virological examination on the day of admittance to the clinic The faeces were collected in Hank's BSS The sample was centrifuged at 10 000 rev/min for 10 minutes (5000 x g) to remove coarse particles The supernatant fluid was used as inoculum for the cultures after pretreatment with G penicillin 200 i u /ml dihydrostreptomycin 200 mg/ml and mycostatin 200 i u /ml

2) Autopsy material from seven kittens aged between 9 and 12 weeks (A 992, A 1113 A 1188 A 855 AG 1, AG 2, AG 3) from the Copenhagen area Three were litter mates and the remaining apparently unrelated cases. There was a history of sudden illness and death in all cases Patho-anatomical lesions localized in the lower small intestine varying from slight congestion to erosions and haemorrhage, were observed In addition there was oedema and hyperaemia of the mesenteric lymph nodes

Jejunum and mesenteric lymph nodes were used for viral isolation The material was prepared as a 10 per cent suspension in Hank's BSS and treated in the same way as the faeces sample described above

Cell culture and virus isolation techniques Primary monolayer cultures of kidney from kittens were prepared according to the method of *Madin et al* (1957) The cells were grown on 10 × 40 mm coverslips in Leighton tubes of 37°C in 2 ml medium. The growth medium consisted of Hank's BSS containing 0 5 per cent lactalbumin hydrolysate, 0 01 per cent yeast extract, 1 0 per cent glucose and 10 per cent calf serum The maintenance medium was 5 ml Earle's medium containing lactalbumin yeast extract and glucose in the same quantity as above and 2 per cent calf serum G penicillin 100 i u /ml dihydrostreptomycin 100 mg/ml and mycostatin 100 i u /ml were used in all media.

The cell cultures were 3 5 days old when used for virus inoculation The growth medium was removed and 0 2 ml of the supernatant fluid was inoculated After adsorption of the virus to the cells for one hour at room temperature maintenance medium was added The tubes were incubated at 37° C Samples were taken from inoculated and control tubes each day from the 1st to the 5th day These were fixed in methanol and stained with haematoxylin eosin

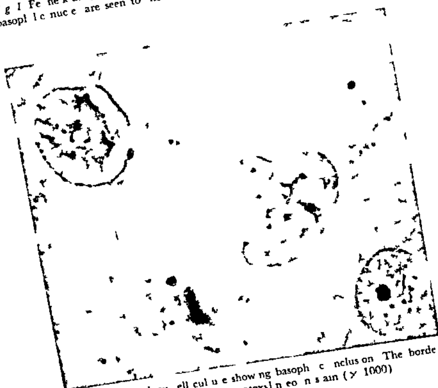
Inoculation of cats Inoculation experiments were made on cats using culture material Five 7 week old kittens and three 8 week-old kittens were inoculated intraperitoneally with 0 5 ml culture fluid of the third passage of the isolation from faeces of the live cat described above

RESULTS

Isolation of virus in tissue cultures Agents which produced identical changes in *feline*



F g I Fe ne k dney cell cul u e sl ow ng eos noph l c ncl s on bod es Dead basopl l c nuc e are seen to he ght Haematoxyl n eos n s a n (× 640)



F g Fe ne k dney ell cul u e show ng basoph c nclus on The borde of he n lu on s sca oped Haematoxyl n eo n s ain (× 1000)

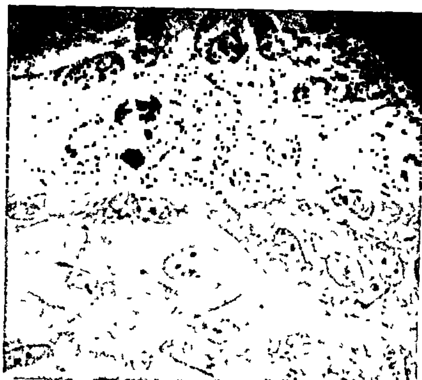


Fig 3 Non inoculated feline kidney cell culture Haematoxylin eosin stain (× 640)

kidney cell cultures were isolated on first passage from the clinical case of panleukopaenia and from jejunum and mesenteric lymph nodes from six out of the seven kittens In a few cases a cytopathic effect was observed in the first passage in unstained preparations 48 96 hours after infection in the form of a roughening and thinning of the cell sheet, with scattered opaque dead nuclei in random patches After a few days' incubation, it was not possible to distinguish between inoculated and control cultures in unstained preparations since the control cultures tended to be roughened and clumpy In the remaining cases no cytopathic changes were observed but in stained preparation the following observation could be made The first evidence of infection observed was enlargement of the nucleoli and rarefaction of the chromatin around them, causing a halo effect Later eosinophilic inclusion bodies occured in the nucleus The chromatin marginated to the nuclear membrane, leaving a clear zone between it and the central eosinophilic inclusion Later the inclusion bodies became more homogenic and changed in colour from eosinophilic to basophilic The periphery of the inclusion was relatively smooth though some had a wrinkled or scalloped border in the late stages (Fig 1-2) The cytoplasm of the affected cells showed retraction as degeneration proceeded Pyknotic nuclei representing the final stage of degeneration appeared in the form of small basophilic-stained cells with wrinkled nuclear membranes (Fig 1) The inclusion was often only evident after prolonged examination of a culture since the inclusions were not uniformly distributed throughout the cell sheet The number of affected cells varied considerably with age of the culture and growth of the cells at the time of inoculation The best time for inoculation was found to be before a full sheet of cells had developed in quickly growing cells with many mitoses. Cultures with slowly-growing cells could not be used The first in-

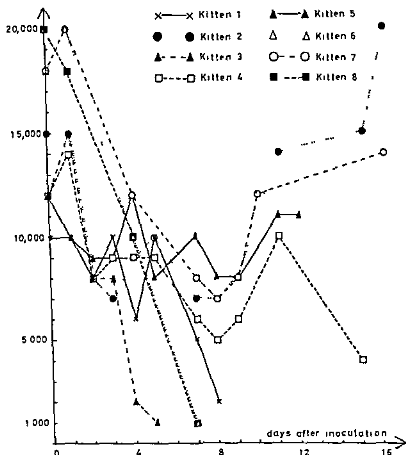


Fig 4 Total leukocyte count after inoculation of kittens with feline panleukopaenia culture fluid The kittens 1, 3 4, 6 and 8 died 9, 6, 15, 7 and 7 days respectively after inoculation Depression, anorexia and diarrhoe were seen in these kittens for 1 to 4 days in the period of maximal leukopaenia.

clusion bodies occurred sometimes 1 2 days after inoculation They persisted for 3 5 days, after which they disappeared gradually, leaving a full sheet of cells Inoculated cultures appeared to be less dense than the control cultures (Fig 3), but the difference was not marked At a later stage, the only observable change in inoculated cultures was accumulation of scattered dead nuclei

Transmission experiment All kittens used appeared clinically normal from birth until inoculation, apart from a mild conjunctivitis and rhinitis observed in three kittens one week before the transmission experiment A few *Toxocara cati* eggs were found on examination of faeces The total leukocyte count was within normal range before inoculation consistently between 15,000 and 17,000/mm³ in five kittens, and between 17,000 and 20,000/mm³ in the kittens with conjunctivitis and rhinitis This condition may be the reason for the higher values in these kittens

There was a gradual decrease in the total leukocyte count during the first 4-9 days after inoculation (Fig 4)

No signs of illness were seen until the period of maximum leukopaenia when five kittens showed acute and severe illness characterized by depression, anorexia, decreased water consumption and diarrhoea. The kittens appeared weak and dehydrated Palpation of the abdomen revealed excess of gas

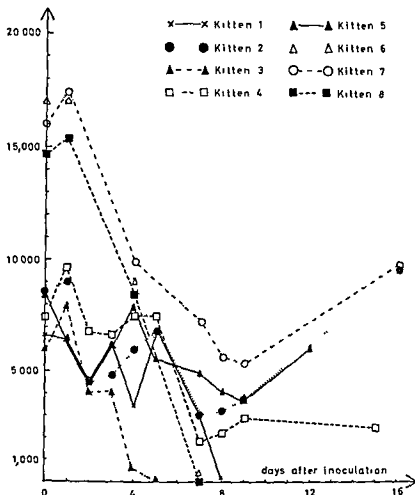


Fig 5 Absolute neutrophil count after inoculation of kittens with feline panleukopaenia culture fluid

and liquid Abdominal pain was evident on palpation Only one kitten had a significant pyrexia (3? ? C and two kittens had subnormal temp rature 36 0° C) Four kittens died 6-9 days after inoculation and one on the 15th day af (1 to 4 days illness No typical manifestation f line s was observed in the three remaining kittens although a slight decrease in total leukocyte count was observed (Fig 4) These kittens were euthanized 12 16 days after inoculation

It was characteristic that both neutropaenia and lymphopaenia occurred (Fig 5 Fig 6) Especially a decrease in neutrophils could be seen During the course of the illness no abnormally immature cells of any of the leukocytes series were found

Using feline kidney cell cultures, attempts were made to isolate the virus from organs (spleen liver, intestines lgl mediastinalis) from three kittens Virus was isolated from the organs of two which died on the 7th day after inoculation of tissue culture material Inclusion bodies identical to those seen in connection with the primary isolation cultures were observed in the cultures

DISCUSSION

Virus isolation was performed from faeces of one cat with symptoms of feline panleukopaenia, and from autopsy material from six out of seven cats with macroscopical lesions of panleukopaenia A cytopathic effect was

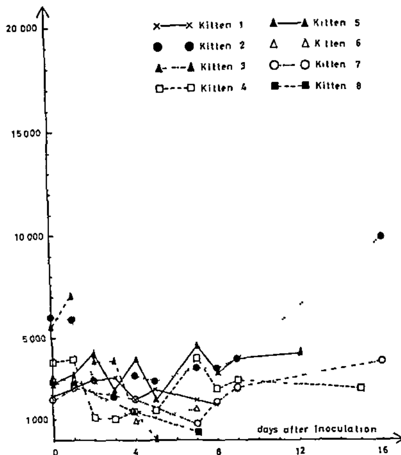


Fig 6 Absolute lymphocyte count after inoculation of kittens with feline panleukopaenia culture fluid

observed in feline kidney cell cultures in a few cases in the form of a roughening and thinning of the cell sheet, with scattered opaque dead nuclei in random patches The virus produced intranuclear inclusion bodies in cell cultures The intranuclear inclusion bodies were only detectable in stained preparations Both the cytopathic effect and the intranuclear inclusion bodies occurred irregularly and was of a transient nature As also observed by *Johnson* (1965) the growth of the virus seemed to depend on high mitotic activity of the cell cultures

Fulminating symtoms of panleukopaenia developed in five of eight kittens in an inoculation experiment Four of these died 6 9 days after inoculation and one on the 15th day after having shown the typical symptoms for 1 to 4 days Clinically, the disease induced in the kittens was acute in nature and identical to naturally occurring panleukopaenia and consistent with the clinical signs described by *Lawrence* & *Syverton* (1938) Haematological changes identical to those in spontaneous disease were also found The haemotological change leukopaenia and agranulocytosis is considered pathognomonic Viral agents causing the same characteristic intranuclear inclusion bodies in cell cultures as the virus used as inoculum were isolated from organs of inoculated kittens It thus seems highly probable that the virus isolated was the cause of panleukopaenia, even if it was not confirmed by serological and histological examina-

tions of the kittens of the transmission experiment

The titre of the virus used in the transmission experiment was not determined owing to difficulty in getting a reproducable titre The different batches of cell cultures varied in their susceptibility, the range obtained was between $10^{?0}$-$10^{4.5}$ $TCID_{50}$/0 1ml For this reason it has been difficult to standardize a serum neutralization test, but work is in progress using a neutralization test according to *Scott et al* 1970

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LIGHT AND ELECTRON MICROSCOPY OF THE EARLY RESPONSE OF *ESCHERICHIA COLI* TO A 6β-AMIDINOPENICILLANIC ACID (FL 1060)

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The morphological response of *E coli* to a new antibiotic, 6β [(hexahydro 1H azepin 1-yl)-methyleneamino] penicillanic acid (FL 1060), has been investigated and compared with the response to benzylpenicillin and ampicillin At high concentrations of FL 1060 (1000 μg/ml) in osmotically stabilized media *E coli* responds in the same way as to penicillins by forming rabbit ears' and spheroplasts At lower concentrations down to the IC50 value (0 02 μg/ml), the cells even in unstabilized media, first become ellipsoidal and later spherical After 2 3 hours lysis of the cells occurs This is rather late, as compared to the early lysis obtained with penicillins Electron microscopical investigations show no characteristic changes in the subcellular pattern. During the second hour of treatment, the bacterial culture contains a considerable number of cells presenting asymmetrical cell divisions. During the same period, nuclear stainings show abnormal nuclear regions with impaired segregation, resulting in chromatine bridges and horseshoe shaped chromatine regions The results support the conception that, on cells in the pre lytic stage, FL 1060 interferes with the balance between lengthwise growth and cell division through cross wall formation

A previous paper (10) described the synthesis and antimicrobial properties of a new group of penicillanic acid derivatives, viz 6β amidinopenicillanic acids It was shown that the antibacterial properties of this class of compounds differ from those of penicillins such as *benzylpenicillin* and ampicillin, which can be regarded as acyl derivatives of 6β amino penicillanic acid

Whereas the penicillins in general are much more active against gram positive than against gram-negative organisms, the reverse is true of the 6β-amidinopenicillanic acids Several of these show a remarkably high activity against *Enterobacteriaceae* whereas their activity against gram-positive organisms is relatively low Thus, one member of the series, 6β-[(hexahydro 1H azepin-1-yl)-methyleneamino] penicillanic acid (FL 1060) was found to be about 500 times more active than benzylpenicillin against *E coli in vitro*, but 250 times less active than benzylpenicillin against a non penicillinase producing strain of *Staphylococcus aureus* (10)

The structures of FL 1060 and benzylpenicillin are shown below

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Requests for reprints should be addressed to *Nina Hellwig Melchior*, Leo Pharmaceutical Products, DK 2750 Ballerup Denmark

$CH_2-CH_2-CH_2 \searrow^{+} N - CH=N$ … (structural formula)

FL 1060

(structural formula)

Benzylpenicillin

Some results of studies on the early morphological response of *E coli* to the influence of FL 1060 and benzylpenicillin are presented in this paper

MATERIALS AND METHODS

Chemicals

Benzylpenicillin was used as the pure sodium salt Ampicillin was used as the trihydrate, and the concentrations were calculated as the anhydrous zwitterion FL 1060 was used as its hydrochloride dihydrate which forms colourless crystals and is readily soluble in water The concentrations were calculated as the anhydrous zwitterion

Bacterial Strain

Escherichia coli (Leo HA) was used as test organism.

Culture Media

The bacte[illegible] own in NIH broth (yeast extract Difco [illegible] asein hydrolysate (pancreatic) 1 5 per [illegible] n chloride 0 25 per cent L-cystine [illegible] with either glucose 0 1 per cent or [illegible] per cent in distilled water), pH 7 1 after [illegible] ng Before electron microscopy the cells [illegible] ntrifuged (1000 g) and resuspended in [illegible] tate peptone broth (YAP) with or without 1[illegible] per cent sucrose (yeast extract Difco 0 3 per cent sodium acetate 0 05 per cent, peptone Difco 0 3 per cent in distilled water)

Microbial Sensitivity

IC50 is defined as the concentrat[illegible] per cent inhibition of a growing ba[illegible] tion in broth After inoculation with 10^4 cells/ml the cultures were incubated at 36° C for 18 hours

Preparations for Light and Electron Microscopy

NIH broth (with 10 per cent sucrose) was used for study of the response of *E coli* to benzyl penicillin or ampicillin and the corresponding control cultures, and normal NIH broth (with glucose) for study of the effect of FL 1060 An overnight culture of bacteria adapted to the medium in question was diluted to allow growth before adding the antibiotic For benzylpenicillin and ampicillin the culture was an overnight culture diluted 1 20 and for FL 1060 a culture diluted 1 150 The experiments were performed at 37° C

For microscopical studies of cell size and form samples of bacteria grown in a series of concentrations were taken every 15 minutes A loopful was placed on a slide and examined directly under a cover glass using Nomarski interference contrast (Reichert) In experiments during which photomicroscopy was carried out, samples were treated with formalin in order to stop any further development or growth, but comparative studies were made to ensure that there were no changes as compared to living samples Exposures were made on Agfa Isopan IF, 17 DIN

For chromosome studies, a loopful of bacteria was spread on a slide and air dried heat fixed hydrolyzed in 1 N HCl at 60° C for 10 minutes and rinsed under tap water The specimen was stained with Giemsa (2 5 ml azure-eosin methylene blue stain solution (Merck), 5 ml phosphate buffer pH 7 0 and 45 ml distilled water) for 18 minutes at 37° C and air dried for a further 15 minutes before microscopy

The time of exposure to the antibiotics in experiments with light and electron microscopy is not directly comparable For the former it is equal to the time of growth at 37° C while for the latter it also includes the time of centrifugation (15 minutes at 1000 g) at room temperature up to the time the cells are resuspended in 10 ml YAP medium Pre fixation was performed immediately after by adding slowly 1 ml of 12 5 per cent glutaraldehyde in barbiturate acetate buffer pH 6 1 to the cell suspension while thoroughly shaking the tube After 30 minutes at room temperature the cell suspensions were centrifuged (1000 g) for 30 minutes and the pellets obtained were enrobed in agar at 45° C (1 5 per cent Noble Agar Difco in barbiturate acetate buff[illegible] pH 6 1) After hardening of the agar, bloc[illegible] 1 mm³ were cut and fixed overnight in [illegible] glutaraldehyde in barbiturate acetate bu[illegible] at room temperature (in some experi[illegible] After a brief wash in barbiturate [illegible] arried out for 2 hours at [illegible] cent barbiturate bu[illegible] 0.1 per

cent YAP medium was added In the experiments with benzylpenicillin sucrose was added to a final concentration of 10 per cent to all reagents used for the fixation procedure After a brief wash in barbiturate buffer pH 6 1 and a one hour treat ment at room temperature in 2 per cent uranyl ace tate in the same buffer (14), the agar blocks with bacteria were dehydrated in alcohol and propylene oxide (9) and finally embedded in Vestopal W (15) After hardening of the blocks sections were obtained on the LKB ultrotome III microtome The sections were post stained for 15 minutes with magnesium uranyl acetate (1) and afterwards for 2 minutes with lead citrate (12) diluted 1 10 with redistilled water Electron microscopy was carried out with a Philips EM 300 electron microscope Exposures were made on Eastman Kodak Fine Grain Release Positive Film Type 5302 at primary magnifications of 1500 and 9000 × and suitable fields were enlarged photographically as desired

RESULTS

Light Microscopy

E coli responded by lysis to the action of penicillins and FL 1060 at concentrations above the IC50, provided that the cells were in the growth phase throughout the time of exposure For benzylpenicillin the time required for this response was 1 1 5 hours in the system described above equivalent to 3-4 generations For practical reasons (in connection with microscopical and turbidometrical investigations), it was convenient to work with a dense population of bacteria *e g* 10 10^9 cells/ml, and the penicillins were therefore added to the growing culture at a time when the number of cells was equivalent to about 5 per cent of an overnight culture However, under the influence of FL 1060 the majority of the cells needed 2 to 3 hours to reach the lytic stage and preliminary experiments showed that a dilution of 1 150 of an overnight culture was needed to give time for the necessary number of generations When FL 1060 was added to a culture of the same density as that described as being convenient for studying the morphological response to benzylpenicillin the cells stopped dividing before they reached the state of lysis (18) The result was the formation of a variety of cell forms Some became ellipsoidal to spherical, others dumb bell to propeller shaped some bean or spindle shaped, and others amoeboid or irregular During growth the tubidity of a culture to which FL 1060 was added increased up to a maximum only slightly below that of a control culture (18) However, since the shape and size of the cells exposed to FL 1060 were totally different from those of control cells the turbidity could not be used as a measure of the number of cells When a culture was allowed to grow for 2 to 3 hours under the influence of FL 1060 as described above, only two morphological forms developed, *viz* ellipsoidal and spherical

The detailed morphological response was followed by viewing single cells growing in a semisolid medium in a perfusion chamber Time lapse cinematography showed that after addition of FL 1060 the cells underwent one or two normal divisions At the next division they no longer elongated but became ellipsoidal The cells still separated and started a new division but it took longer to accomplish the division and they grew still more ellipsoidal After the next extended doubling time (t_d about 1 5 times that of the control), the cells were spherical The division process was now even slower (t_d about three times that of the control) and an equatorial constriction could be seen for a long time Finally the cells lysed The knowledge obtained in a semisolid medium regarding division pattern and changes in size and form of single cells permitted choice of some stages of antibiotic influence in a fluid culture system which were representative of the course of events The fluid culture system gave a picture of the range and average response of the population

After treatment with benzylpenicillin or ampicillin there were three stages of morphological response At a low concentration around the IC50 value, *E coli* formed elongated and filamentous multinuclear cells with or without bulges (Figs 1 and 2) These cells were osmotically stable, but when the penicillin level was raised above the IC50, it was necessary to add an osmotic stabilizing agent to be able to see the spheroplast forma


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tion At a concentration slightly above the IC50 the penicillin caused the bacteria to form rod shaped cells with small protrusions (Fig 3) Most cells did not develop spherical forms but lysed at that stage When the concentration was raised to 5-10 times the IC50 most cells formed "rabbit ears" growing out into spherical forms (Figs 4 and 5) These showed pronounced plasmolysis before they finally lysed in the sucrose containing medium

The morphological response to FL 1060 was shown to be independent of the concentrations around the IC50 and up to 10,000 times that concentration In the fluid culture experiments, we used 0 2 μg/ml of the compound corresponding to 10 times the IC50 After two or three divisions the rod formed *E coli* (Fig 7) stopped growing lengthwise and instead became thicker (Fig 8) After three to four divisions, the *E coli* cells were ellipsoidal with pointed ends (Fig 9) At any time after that, some of the cells lysed with a sudden burst (Fig 10) The remaining had a very extended doubling time and grew as spherical cells increasing in size (Figs 11, 12 and 13) Only when the concentration was increased to 50,000 times the IC50 did some cells show the typical penicillin response, i e 'rabbit ear' formation after ½ 1 hour in sucrose containing medium

The configuration of the nuclear regions was followed during the same experiments After 1½ hours the *E coli* control cells divided rapidly with two to four nuclei per bacterium (Fig 14) The bacteria exposed to FL 1060 became thicker and slightly ellipsoidal after 1 hour, but hardly any changes were seen in the nuclear regions (Fig 15) After 1½ hours, when the *E coli* cells were clearly ellipsoidal, the nuclear region was somewhat more dense as if it contained more DNA than normal (Fig 16) Chromatine bridges could be seen between the daughter nuclei, and these tended to separate in a plane perpendicular to the equatorial plane of the parent cell This was still more pronounced after 2 hours (Fig 17), at which time the nuclear regions became very dense and horseshoe shaped After 3 hours these regions became disorganized (Fig 18), and there seemed to be a larger amount of soluble nucleotides i e with the same 10 minutes of washing the chromosomes did not contrast well against the cytoplasm which was still stained intensively

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Fi
Fi
Fi
Fi

Fig 6 Control cells grown for 1½ hours

E coli cells grown in N I H broth with no sucrose added The early response to FL 1060 0 2 μg/ml is shown.

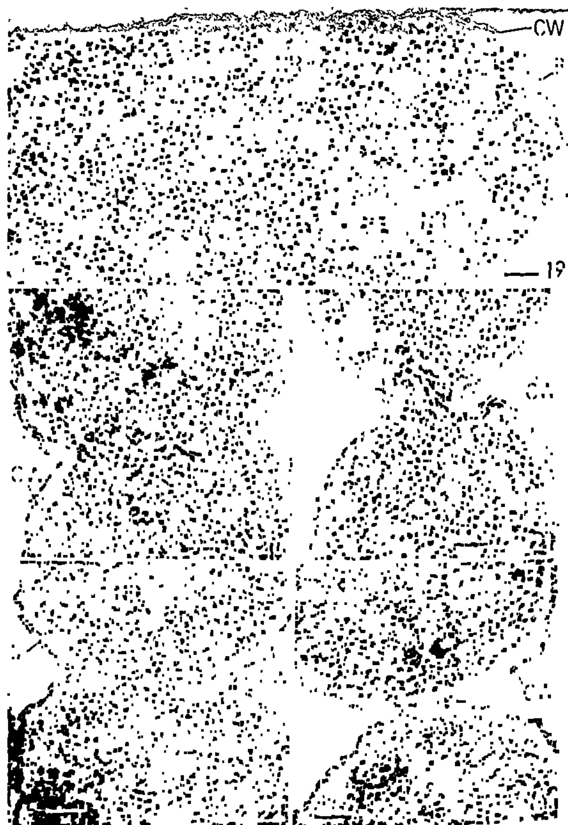
Fig 7 Control cells grown for 1¾ hours
Fig 8 FL 1060 for 1 hour
Fig 9 FL 1060 for 1¼ hours
Fig 10 FL 1060 for 1½ hours
Fig 11 FL 1060 for 1¾ hours
Fig 12 FL 1060 for 1¾ hours
Fig 13 FL 1060 for 2 hours

Giemsa stained chromosomes of *E coli* cells grown under the influence of 0 2 μg/ml FL 1060 in N I H broth with no sucrose added

Fig 14 Control cells grown for 2 hours
Fig 15 FL 1060 for 1 hour
Fig 16 FL 1060 for 1½ hours
Fig 17 FL 1060 for 2 hours
Fig 18 FL 1060 for 3 hours

Electron Microscopy

Previous electron microscopical investigations of the E coli cell envelope have demonstrated that it consists of an inner triple layered plasma membrane, and a cell wall complex consisting of an outer triple layered membrane and an intermediate layer identified as the murein skeleton (2) The control *E coli* showed this characteristic gram negative cell wall profile (Fig 19) The size of the *E coli* was measured from survey electron micrographs and the length and diameter of individual cells were found to be


CW
19

about 1 7-2 8 μm and 0 6 0 8 μm, respectively The outline of the cell wall of the control *E coli* was wavy and small protrusions or "blebs" were seen on the outer membrane (Fig 19) Apposed to the inner leaflet of this membrane a dense line, the intermediate layer, was observed This was seen best in bacteria which showed slight plasmolysis (Figs 22 and 23) The total thickness of the outer membrane and the intermediate layer was about 12-13 nm, the intermediate layer probably amounting to about 2-3 nm Beneath the intermediate layer, and in some places apart from it because of the plasmolysis, the plasma membrane was seen as a triple layered membrane of the same dimension as the outer membrane, 8-10 nm (Figs 22 and 23) Numerous clusters of ribosomes measuring 11-15 nm were seen in the greyish cytoplasmic matrix (Fig 19) The nuclear regions appeared in the form of light irregular patches in which the dark filamentous DNA containing structures could be seen (Fig 19)

Some interesting details were obtained from the study of dividing cells from cultures with and without sucrose No plasmolysis occurred when *E coli* was grown without sucrose, and during the division process all layers of the envelope participated in the concentric invagination process without any visible separation of the layers, *i e* no real septum formation was seen (Figs 20 and 21) With 10 per cent sucrose in the medium, a moderate plasmolysis occurred and the plasma membrane was separated from the intermediate layer (Fig 22) The cell division proceeded as a concentric inward growth of the plasma membrane (Fig 22), and when opposite sides met they fused and formed two separate plasma membranes (Fig 23) The distance between these two membranes was about 55 nm, and the space was filled with a homogeneous greyish material into which the outer membrane was seen to invaginate (Fig 23)

After 3 hours of benzylpenicillin treatment the typical "rabbit ear" cells were seen together with some spherical cells, as shown in the survey micrograph (Fig 24) Some of the cells had already lysed as seen at the bottom of the picture Higher magnifications of about 20 different "rabbit ear" forms were studied, revealing some morphological characteristics In all the cells examined, a pronounced plasmolysis was present only on the rod shaped parts In the middle of the bacterium where the characteristic bud had formed no plasmolysis was ever seen and all parts of the cell envelope were lying close together (Fig 25) Another characteristic response to treatment with benzylpenicillin was the formation of spheroplasts For the purpose of this study, this term is used for cells of spherical shape with some material missing in the intermediate layer region (Fig 26) The outlines of the outer membranes of the spheroplasts were quite smooth with only a few "blebs" (Fig 26) In some places there was moderate plasmolysis The overall thickness of the cell envelope was measured to

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For the electron micrographs the following abbreviations are used

B — blebs
CW = cell wall
IL = intermediate layer of cell wall
LC — lysed cell
NR nuclear region
OM = outer membrane of cell wall
PL = pre lysed cell
PM = plasma membrane
R = ribosome
RE = "rabbit ear
S = spheroplast
V = vacuole

The bar represents 1 μm in the micrographs with low magnification and 0 1 μm in micrographs with high magnification

Fig 19 Control cell grown for 2 hours in N I H broth 90 000 ×
Fig 20 Control cells grown for 1 hour in N I H broth 90 000 ×
Fig 21 Control cells grown for 1 hour in N I H broth 90 000 ×
Fig 22 Control cells grown for 2 hours in N I H broth with 10 per cent sucrose 90 000 ×
Fig 23 Control cells grown for 2 hours in N I H broth with 10 per cent sucrose 90 000 ×



25
PM
CW
OM
V
PM
NR

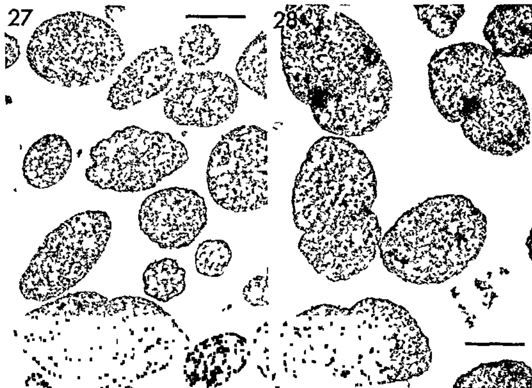


Fig 27 and Fig 28 Cells grown for 1½ hours in N I H broth under the influence of FL 1060, 0 2 µg/ml 15 000 ×

about 20 nm the outer membrane and the plasma membrane measuring 9 10 nm No distinct intermediate layer could be found In some of the spheroplasts vacuoles were present in the peripheral part of the cytoplasm (Fig 26) The nuclear regions had apparently expanded and the filamentous DNA containing structures were seen clearly (Fig 26)

The morphological response to treatment with FL 1060 progressed through different stages, as seen by light microscopy (Figs 7-13) After treatment for 1-1½ hours most of the cells had attained an ellipsoidal form with pointed ends The lengthwise growth was no longer pronounced and instead the bacteria had increased in thickness (Fig 27) From a count of about 60 dividing cells in survey electron micrographs, it was found that about 50 per cent showed an asymmetrical division, while the rest showed a concentric invagination of the cell envelope (Fig 28) These two types of division are shown in higher magnifications in Figs 29 and 30 No difference in ultrastructure could be observed either between the individual layers of the envelopes of these two types or those of the control cells (compare Figs 29, 30 and Figs 20, 21) However, when comparing symmetrically dividing treated cells with dividing cells of the controls, it was obvious that the degree of curvature at the division sites was much less in treated cells (Fig 29) than

Fig 24 Cells grown for 3 hours in N I H broth with 10 per cent sucrose under the influence of benzylpenicillin 100 µg/ml 15 000 ×

Fig 25 Detail from a rabbit ear shaped cell grown under the same conditions as for Fig 24 90 000 ×

Fig 26 Detail from a spheroplast grown under the same conditions as for Fig 24 90 000 ×


CW
NR
29
R
30

in control cells (Figs 21 and 22). In the interior of treated cells, the number and distribution of ribosomes appeared to be normal, as did the nuclear regions of these cells In cells showing the asymmetrical division, the ribosomes appeared to be clustered at the invagination site (Fig 31) No sign of invagination was seen on the diametrically opposed cell wall, and all three layers of the cell envelope seemed to participate in the asymmetric ingrowth of new cell wall material (Figs 30 and 31).

After treatment for 2 hours, some very big spherical cells with diameters up to 3 4 μm were present In spite of the great increase in the volume of the cells, the cell wall had preserved the wavy outline of its outer membrane and quite a few "blebs" were visible (Fig 32). The outer membrane and the plasma membrane were both 9-10 nm in thickness, whereas the intermediate layer could not be measured exactly The size of ribosomes had apparently increased to 15-20 nm instead of the 11-15 nm found in the control cells The nuclear regions had swelled and seemed to be scattered throughout the cytoplasm of the cell (Fig 32)

When the treatment with FL 1060 was continued up to 3½ hours nearly all cells lysed Fig 33 shows ghosts of two lysed cells and a bacterium in a pre-lysis state In this state some vacuoles often appeared in the bacteria, and these vacuoles were surrounded by a unit membrane resembling the plasma membrane A part of such a bacterium is seen in Fig 34 The cell wall outline was

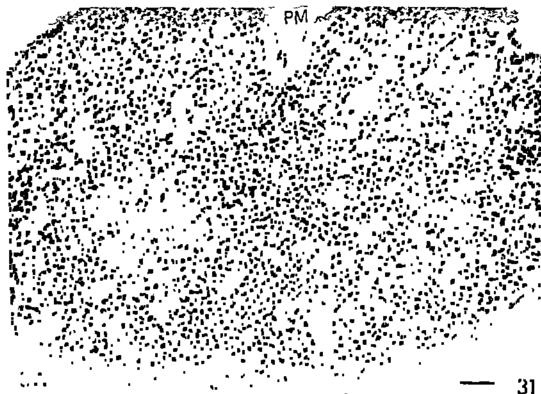


Fig 29 Detail of a concentric invagination of the cell envelope of a dividing cell from the same culture as for Figs 27 and 28 90,000 ×.

Fig 30 and Fig 31 Detail of an asymmetrical invagination of the cell envelope of a dividing cell from the same culture as for Figs 27 and 28 90,000 ×

26*


32
CW
R
PM
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now more even and very few 'blebs' were seen Due to moderate plasmolysis the intermediate layer and the plasma membrane were separated The thickness of the outer membrane and the apposed intermediate layer was about 14 nm, the intermediate layer amounting to 4 5 nm The plasma membrane measured 9-10 nm Compared with the dimensions of these layers in the controls, the intermediate layer of the treated cells thus seemed to have increased somewhat in thickness, whereas the other two structural parts appeared to be unaltered

From experiments with benzylpenicillin treatment the effector site is known to be situated in the cell wall, probably in the intermediate layer region (2) A series of micrographs of cells treated with benzyl penicillin (Fig 36) and FL 1060 (Fig 37) were mounted together with a control cell (Fig 35) in order to give a direct comparison of the effect of these compounds on the cell wall No structural difference could be seen between the cell walls of the control cells and the cells treated with FL 1060, except perhaps for the slight increase in thickeness of the intermediate layer mentioned above In contrast to this, measurements on organisms treated with benzylpenicillin showed that the murein or intermediate layer had become thinner or had more or less vanished completely (Fig 36)

Fig 32 Cell grown for 2 hours in N I H broth under the influence of FL 1060 0 2 μg/ml Only part of a big spherical cell is shown 90 000 ×

Fig 33 Cells grown for 3 hours in N I H broth under the influence of FL 1060 0 2 μg/ml 15 000 ×

Fig 34 Cell grown under the same conditions as for Fig 33 A part of a cell in the pre lysis state is shown 90 000 ×

Fig 35 Part of a control cell grown for 2 hours in N I H broth with sucrose 90 000 ×

Fig 36 Part of a cell grown for 3 hours in N I H broth with sucrose under the influence of benzyl penicillin 100 μg/ml 90 000 ×

Fig 37 Part of a cell grown for 2 hours in N I H broth under the influence of FL 1060, 0 2 μg/ml 90 000 ×

DISCUSSION

The effect of a new penicillanic acid derivative, FL 1060, on the growth and morphology of *E coli* has been studied When comparing the effect of this compound on cells with that of penicillins, some similarities and several dissimilarities were observed Both types of antibiotics exert their bactericidal activity only on cells in the growth phase, and the cells must be in active growth throughout the whole time of exposure to reach the lytic stage (18) Exposed cells develop different morphological forms in the pre lytic phase depending on the concentrations used

At concentrations below the IC50 value, penicillin shows its effect on the division septum only, and the bacteria form long unseptated filamentous cells (Fig 1), as already reported by *Lederberg* (8) At penicillin concentrations around the IC50, the bacteria form filaments with lateral bulges (Fig 2), which according to *Schwartz et al* (16) are filled with ribosomes *Weidel* & *Pelzer* (19) have shown that the rigid macromolecule murein determines the shape of the bacterial cell In the present experiments, the filamentous shaped cells with or without bulges could be produced in normal medium without any osmotic stabilization This is in accordance with the observation (16) that there are no breaks in the murein sacculi of these cell forms To produce the so called "rabbit ears" and spheroplasts (Figs 4 and 5) the bacteria must grow in osmotic stabilized media with concentrations of penicillin well above the IC50 *Schwartz et al* (16) could show that under such conditions the murein molecule splits sharply into two halves

Electron micrographs of 'rabbit ears' show the plasma membrane lying closely attached to the outer membrane in the bulges, but in general there still seems to be some intermediate layer left (Fig 25) However, in the spheroplasts (Figs 26 and 36), the intermediate layer can hardly be observed In pa-

rallel experiments without sucrose, the cells reach the lytic stage quite quickly

FL 1060 can induce the same early formation of "rabbit ears" and spheroplasts as penicillins. However, this response can only be achieved at very high concentrations (1000 $\mu g/ml$). *At all lower concentrations down to the IC50 value of 0.02 $\mu g/ml$ the cells* become ellipsoidal to spherical, irrespective of the variations in concentration in this broad range. No filamentous forms are produced at subinhibitory concentrations. The fact that the ellipsoidal and spherical cells can be produced in media without sucrose would indicate that there are no breaks in the murein layer. Electron micrographs show no decrease in the thickeness of the intermediate layer of the spherical cells developed under the influence of FL 1060 (Figs. 34 and 37). The light microscopical investigations show that these cells do not protrude out of a rod shaped cell but that the entire cell changes into the spherical form. The spherical cells reach the lytic stage within 2-3 hours.

There is a very delicate interplay between the various cell components participating in *the division of a cell.* Cell growth can briefly be described as initiation, cell component synthesis, cell elongation and cell division, with some stages overlapping each other. Cell component synthesis includes DNA, RNA and protein syntheses and the accumulation of prote[illegible]n [illegible] acts as an initiator of chromosome replica[illegible] (3, 4). In a normal *E. coli* cell m[illegible]h takes place by cell elong[illegible] cell division through cross w[illegible] 22 and 23). According to *H*[illegible] (5) there is a distinct sep[illegible]e [illegible]o growth forms *i.e.* at the tim[illegible] division the synthesis of cell wall mate[illegible]n[illegible]ly diverted into *the formation of th*[illegible] cross wall. The time from the first sign [illegible] constriction until the daughter cells are separated is quite short.

Under the experimental conditions used in this study, growing *E. coli* cells must be exposed to FL 1060 for almost one hour to achieve the ellipsoidal form. In this period the DNA, RNA and protein syntheses continue as in the control cells (7, 11). Our electron microscopical studies have shown that the cell components such as ribosomes and chromatine material show a normal distribution and cell wall components have a normal appearance. The optical density also increases but nevertheless the viable counts show a marked decrease (18).

During the second hour of treatment the cells grow spherical and bigger and a large number of cells with a central constriction can be seen (Fig. 12). The total cell mass measured as optical density still increases (18). Time lapse cinematography of cells grown in a perfusion chamber shows that exposure to FL 1060 increases the doubling time by a factor of 1.5-3. Consequently FL 1060 must somehow interfere with the mechanism of cell division.

Cell elongation and cell division are normally distinctly separate processes and the ingrowth of cross wall occurs at right angles to the long axis of the cells (Figs. 22 and 23). The morphology of the cell is thus characterized by a rather narrow transitional zone between the cross wall and the cylindrical part of the cell. Treatment with FL 1060 results in a considerable number of ellipsoidal cells (Figs. 27 and 29), and the transitional zone has now become the dominating part of the cell wall. The cell wall elongation and cross wall formation thus seem to be unbalanced and this will further influence the cell division processes. This point of view is supported by the fact that a considerable number of cells with asymmetrical divisions are observed (Figs. 30 and 31).

Normally the completion of a round of chromosome replication is followed by chromosome segregation. The completion of replication is a necessary condition for cell division but the *replicated* chromosome has to be segregated to make cell division possible. The chromosomes of bacteria are presumed to be attached to the cell membrane and daughter chromosomes to be segregated through the growth of the membrane (3, 6, 13).

The segregation of chromosomes in FL1060 treated cells is affected, as can be seen by Giemsa staining. The nuclear regions first look more dense (Fig 15) and later some cells show horseshoe shaped regions and chromatine bridges (Figs 16 and 17). Chromatine bridges have been observed by *Smith* & *Pardee* (17) who suggested that such bridges could physically block completion of septum formation. The abnormal nuclear regions observed in the present study might be a further cause of the increased number of cells with asymmetrical divisions.

The authors wish to thank Miss *Bente Gram* most cordially for skillful help in growing cultures and preparing specimens for this study. We also wish to express our gratitude to Mrs *J Berg* for excellent assistance in sectioning and electron microscopy, and Miss *A G Overgaard* and Mr *Laursen* for expert handling of the photographic material.

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BACTERIOPHAGE TYPING OF *MYCOBACTERIUM RANAE (FORTUITUM)*. THE CORRELATION OF LYSIS BY MYCOBACTERIOPHAGE BK4 AND INOSITOL UTILISATION

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Forty eight strains of *Mycobacterium ranae* (Bergey et al.) have been phage typed with various dilutions of a suspension of mycobacteriophage BK4. A close correlation between lysis by 5 × 10 or less Plaque Forming Units (P.F.U.) of the phage and inositol utilising activity has been found. Strains that utilise inositol are lysed by 5×10^2 or less P.F.U. whereas strains that are unable to utilise inositol require 5×10^4 or more P.F.U. for lysis. In one group of strains of *M. ranae* the inositol utilisation is greatly reduced and only detectable by a sensitive technique. This is considered to be due to permeability factors rather than a defect in the inositol locus.

The occurrence of bacteriophage in the genus *Mycobacterium* was first described by *Penso & Ortali* (1949). Subsequently the possibility of using bacteriophage typing as a system for mycobacterial identification at the species level has been investigated by several authors (*Redmond* 1963, *Redmond & Ward* 1966, *Juhasz & Bönicke* 1965, *Baess & Weis Bentzon* 1969). In addition the subdivision of *Mycobacterium tuberculosis* by bacteriophage typing has been studied (*Froman et al.* 1954, *Tokunaga et al.* 1968) and introduced as an aid to epidemiological studies (*Baess* 1969, *Bates & Mitchison* 1969). However no reports of correlation between phage typing and other methods of sub-specific typing in the mycobacteria are available.

The species *Mycobacterium ranae* (*fortuitum*) has been shown to contain well defined sub specific types. *Bönicke* (1965) divided the species into three biotypes based on the utilisation of two alcoholic sugars inositol and mannitol. *Stanford & Gunthorpe* (1969) described four serotypes determined by the demonstration of cytoplasmic antigens by the agar gel immunodiffusion technique. Subsequently a fifth serotype (serotype V) has been described in a recent study (*Pattyn et al.* in preparation). The results of this same study have shown that there is a correlation between the agglutination and immunodiffusion serotypes and typing by delayed hypersensitivity in guinea pigs and the biotypes of *Bönicke*. As shown in Table 1 the immunodiffusion serotypes II, III and IV form a biochemically related cluster able to utilise both inositol and mannitol (biotype C). Serotype V strains are unable to utilise inositol

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TABLE 1 *Correlation of the Immunodiffusion Serotypes and Biotypes of* M ranae *Based on the Utilisation of Carbohydrates and the Splitting of Acetamide*

Serotype	Biotype	Gordon's carbohydrate row						Acetamide
		GL	IN	MT	MN	SO	TR	
I	A	+	—	—	+	—	+	+
II	C	+	+	+	+	+	+	+
III	C	+	+	+	+	+	+	+
IV	C	+	+	+	+	+	+	+
V	B	+	—	+	+	+	+	—

GL = Glucose, IN = Inositol, MT = Mannitol, MN = Mannose, SO – Sorbitol, TR = Trehalose

(biotype B) and serotype I strains are unable to utilise either sugar (biotype A)

The relationship of bacteriophage typing to subdivision by serological and biochemical techniques is currently being investigated In this study the relationship of typing with mycobacteriophage BK4 to inositol utilisation is reported

MATERIALS AND METHODS

Mycobacterial Strains

Forty-eight strains of *Mycobacterium ranae* were obtained from Dr *J L Stanford* These strains were serotyped by the method of *Stanford & Gunthorpe* (1969) and biotyped according to their production of acid from various carbohydrates (*Gordon & Smith* 1953, *Bönicke* 1965) They were also tested in the Carbohydrate Nitritase Row (*Bönicke & Kazda* 1970) in which the various carbohydrates are tested for their ability to act as electron donors for the breakdown of nitrite ions by the bacteria

It has been found that the Carbohydrate Nitritase Row may be rendered much more sensitive and able to detect small amounts of enzyme activity by diluting the Sodium nitrite solution to 1/100 th of the standard dilution i e to 0 2 mmg/ml (personal observation) Even at this dilution the difference between positive and negative results is clearly distinguishable This test will be referred to as the Modified Carbohydrate-Nitritase Row

Mycobacteriophage

The mycobacteriophage BK4 and its propagating strain, F 656, were obtained from Dr *Inga Baess* of the Statens Seruminstitut, Copenhagen The phage was propagated in liquid medium by the method of *Jones & White* (1968) and separated from the host strain by membrane filtration (pore size 0 22 mu)

TABLE 2 *The Correlation of Lysis by 5 × 10⁵ and 5 × 10³ P.F U of Phage BK4 and Inositol Utilisation as Determined by Three Techniques*

Serotype	No strains tested	Lysis		Inositol utilisation		
		5×10^5	5×10^3	Gordon	C N R.	M C N R.
I	6	+	+	—	—	+
I	8	+	—	—	—	—
II	8	+	+	+	+	+
III	1	+	+	+	+	+
IV	2	+	+	+	+	+
IV*	1	—	—	+	+	+
V	7	+	—	—	—	—

Gordon = Acid production from Inositol (*Gordon & Smith* 1953)
C.N R. = Carbohydrate Nitritase row (*Bonicke & Kazda* 1970)
M C N R = Modified C N R. described in test
The type IV strain marked with an asterisk carries a phage which renders it immune to superinfection by other phages

TABLE 3 *Lysis of a Group of Twenty Nine Serotype I Strains of M. ranae by Various Dilutions of Phage BK4. The Figures Refer to Plaque Forming Units Estimated on Strain No 843. Table also Shows Inositol Utilising Activity Detected by the Modified Carbohydrate Nitritase Test (Described in Text)*

Strain no	Lysis by phage BK4						Inositol MCNR
	5×10^5	5×10^4	5×10^3	5×10^2	5×10	5	
108Ro	+	+	+	+	+	—	+
431	+	+	+	+	+	+	+
599	+	+	+	+	+	+	+
657	+	+	+	+	—	—	+
843	+	+	+	+	+	+	+
844	+	+	+	+	+	+	+
800	+	+	+	+	+	+	+
45	+	+	+	+	+	—	+
6	+	+	+	+	+	+	—
82	+	+	+	+	—	—	—
4	+	+	+	—	—	—	+
80	+	+	—	—	—	—	—
81	+	+	—	—	—	—	—
83	+	+	—	—	—	—	—
92	+	+	—	—	—	—	—
79	+	+	—	—	—	—	—
443	+	+	—	—	—	—	—
490	+	+	—	—	—	—	—
491	+	+	—	—	—	—	—
576	+	+	—	—	—	—	—
621	+	+	—	—	—	—	—
634	+	+	—	—	—	—	—
676	+	+	—	—	—	—	—
679	+	+	—	—	—	—	—
680	+	+	—	—	—	—	—
799	+	—	—	—	—	—	—
R106	+	—	—	—	—	—	—
R111	+	+	—	—	—	—	—
681	+	—	—	—	—	—	+

Phage Titration and Typing

For the purposes of phage titration and subsequent phage typing a soft agar overlay technique was used. 0.3 ml of a 24 hour culture of the test strain in nutrient broth was added to 7 ml of molten 0.7 per cent nutrient agar + 0.002M calcium chloride at 45° C and mixed well. The mixture was poured onto a layer of 1 per cent nutrient agar in a Petri dish, allowed to solidify and dried at 32° C for two hours.

Bacteriophage suspensions were spotted onto the surface of the soft agar overlay by means of platinum loops delivering 0.003 ml. The plates were examined for plaque formation daily for four days.

Phage suspensions were titrated by spotting 10 fold dilutions onto an indicator strain (collection no 843, received originally from the Statens Serum Institut, Copenhagen, no 516). This strain was used as a reference strain for the estimation of Plaque Forming Units (P.F.U.).

Phage suspensions were adjusted to contain 15 × 10^7 P.F.U./ml, i.e. 5 × 10^5 P.F.U. per loopful. For the estimation of lytic titres 10 fold dilutions of this suspension were made.

All phage typing was performed twice using separately prepared batches of phage suspension.

In a preliminary study representative strains of the five serotypes were phage typed using 5 × 10^5 and 5 × 10^7 P.F.U. Subsequently a group of 29 serotype 1 strains were tested using a series of 10 fold dilutions of the phage suspension.

RESULTS

Table 2 summarises the results of the phage typing of representative strains of the five serotypes.

The results show a close association between lysis by the given phage concentrations and inositol utilisation. Thus there is a di

stinction between the serotype II, III, IV group (biotype C) and serotype V (biotype B) The only exception in the above groups was a type IV strain that was not lysed by phage BK4 Subsequently this strain was shown to carry a phage of its own which rendered the host immune to a group of phages including phage BK4

The serotype I strains were divisible into two groups The first group was lysed by 5 × 10^3 P F U and possessed slight inositol utilising activity as detected by the Modified Carbohydrate-Nitritase test whereas the second group showed neither visible lysis at this phage concentration nor inositol utilising activity

Table 3 shows the results of phage and carbohydrate typing in the group of twenty nine serotype I (biotype A) strains 10 strains were lysed by 5 × 10^2 or less P F U and of these 8 (80 per cent) showed inositol utilising activity 18 strains were lysed only by 5 × 10^4 or more P F U and only one of these possessed inositol utilising activity (strain no 681) One strain (no 4) was inositol positive and was only lysed by 5 × 10^3 or more P F U

DISCUSSION

The results of these studies show a close and significant correlation between lysis by low concentrations of bacteriophage BK4 and the possession of detectable inositol utilising activity Furthermore the difference in lytic titres of phage for the two phage types of Serotype I is 100 fold or more i e from 5 × 10^2 or less P F U to 5 × 10^4 or more P F U The exception to this grouping was an inositol positive strain that was lysed by 5 × 10^3 or more P F U This strain differs from the other serotype I strains by the possession of an additional antigenic line, the significance of which is unknown

Evidence is available that some strains in the serotype II, III IV group are progenitor strains from which serotypes I and V are derivable at a low frequency (*Grange & Stanford*, unpublished data) The change to serotype I involves a considerable change in cell wall structure as evidenced by changes in colonial morphology, lipid chromatographic pattern and agglutination serotype Thus the apparent very slight inositol utilising activity of these strains may be due to permeability factors rather than a mutation directly affecting inositol metabolism Some serotype I strains, however, show a total irreversible loss of inositol utilisation On the other hand the change to serotype V involves the constant loss of inositol utilisation together with the loss of acetamidase activity and the loss of one or more cell wall lipids but without change in agglutination serotype As these changes have so far proved to be irreversible they might be due to deletional mutation

It is therefore postulated that the genetic determinant of a factor affecting the interaction of host and phage is closely associated with the determinant of inositol utilisation and that the two loci may be lost together at a high frequency by deletion

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THE IMMUNOCHEMISTRY OF *STAPHYLOCOCCUS AUREUS* MUCOPEPTIDE

I Antigenic specificity of the peptide subunits

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Antigenic determinants of *Staphylococcus aureus* mucopeptide and corresponding antibodies in rabbit antiserum have been studied using synthetic peptides in inhibition of indirect haemagglutination and immunoadsorbent techniques Results are presented to show that at least 3 determinants are present in the peptide subunits The pentapeptide L Ala-γ D Glu L Lys D Ala D Ala exhibits 2 determinants one of which is located to the C terminal D Ala D Ala and the penta glycine bridge one determinant

The immunogenicity of the mucopeptide (peptidoglycan) structures of Gram positive cocci is well documented (16, 1, 11, 3, 8, 9, 4, 18, 10, 21, 22, 6) and antibodies directed against the polysaccharide backbone (glycan chain) (16, 3, 9, 4, 18) and the peptide subunits (16 11, 8, 4, 10 21) have been demonstrated Precipitin reaction studies of antisera against Streptococcus Group A-variant (21, 22) revealed the presence of antibodies against the pentapeptide (L-Ala γ D Glu L-Lys D Ala D Ala) besides antibodies to the polysaccharide backbone Inhibition studies indicated that the antibodies were directed against the COOH terminal portion of the pentapeptide and that the terminal D alanyl D alanine is the immunodominant site of the mucopeptide However the indirect haemagglutination technique has indicated that the reaction between *Staph aureus* mucopeptide and antisera is complex (6) For instance, there seem to be at least two antigenic determinants in the peptide moiety

In the present study synthetic peptides with amino acid sequences related to the native peptide subunits of *Staph aureus* mucopeptide have been used to reveal the number and positions of antigenic determinants

MATERIALS AND METHODS

Isolated antigenic materials Crude protein A was prepared from *Staph aureus* strain Cowan I as described in (5) Mucopeptide from the same strain was prepared, and examined by circular paper chromatography and by gas chromatography, as previously described (13)

Synthetic materials The peptides P_1 pentapep- [illegible] thetic schedule and analyses of the products are described in (21) A pentaglycine preparation (P_4) was purchased from Fluka AG, and N acetyl D alanine (NAc D Ala) from Sigma

Antisera Antisera to *Staph aureus* strain Cowan I (Cowan I serum) were raised in rabbits by intravenous injection of formalin killed bacteria (15)

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another were observed For one of the Cowan I sera tested the inhibitory capacity of P_2 was estimated to be 156 µg/ml or 0 3 µmole/ml, i e 0 08 µmole of the peptide inhibited agglutination of sensitized TSE in 0 25 ml of the serum (diluted to 8 times its haemagglutination titre) Corresponding figures for the pentapeptide (P_1) were 2 µmole/ml and 0 5 µmole The other preparations tested for inhibition were without effect at concentrations of 10 mg/ml or less

The 4 peptides coupled to activated Sepharose the immunoadsorbent columns I (coupled P_1) II (coupled P) III (coupled P_3) and IV (coupled P_4) were all found to adsorb protein material with activity for Cowan I mucopeptide, as judged by agglutination of mucopeptide and indirect haemagglutination Immunoelectrophoretic analysis using goat antiserum to rabbit serum showed that the isolated materials were pure IgG The IgG material isolated on one column was tested for adsorption to the three other columns (Table 2) The fractions eluted from II and III were completely adsorbed to column I, whereas none of the fraction from column IV was adsorbed to the other columns Some of the IgG fraction isolated on I was adsorbed to II, and the remaining part to III None of the material isolated on column I was adsorbed to IV, and no adsorption was observed when the IgG fractions from II and III were tested on III and II respectively These results were verified upon exhaustion of a serum sample on one column followed by testing on the other columns (Table 3) A serum sample exhausted for material adsorbing to column I showed further activity for IV only, whereas a serum sample completely adsorbed on II still showed activity for the 3 other columns However, a further adsorption of this serum sample on III resulted in activity for column IV only The same result was obtained when another serum sample was first adsorbed on column III (still reacting with the 3 other columns) and then on II The IgG fraction isolated on column IV seemed to be specific for P_4 (Tables 2 and 3) A serum sample

TABLE 3 *Activity of Serum Samples after Exhaustion on Various Columns*

Serum exhausted on column with coupled	Test-columns			
	I	II	III	IV
L Ala γ D Glu L-Lys-D Ala D Ala (I)		—	—	+
L-Ala-γ D Glu L-Lys D Ala (II)	+		+	+
Gly L Ala D Ala D Ala (III)	+	+		+
$(Gly)_5$ (IV)	+	+	+	

\+ adsorption
— no adsorption

TABLE 4 *Minimal Concentrations (µg/ml) of Various IgG Fractions Effecting Haemagglutination* and Agglutination of Mucopeptide*

IgG fractions isolated on column with coupled	Haemaggl	Aggl
L Ala γ-D Glu L-Lys D-Ala D Ala	0 4	6
L Ala γ D Glu L Lys D Ala	0 2	12
Gly L Ala D Ala D Ala	3 4	45
$(Gly)_5$	1 4	50

* haemagglutination of TSE sensitized with crude protein A

exhausted on columns I and IV still agglutinated mucopeptide. Total concentrations of various IgG fractions were found to be in the range of 300 µg/ml of serum (anti P_4) to 700 µg/ml (anti P_1). Due to the relatively small quantities isolated and the occurrence of some denaturation during dialysis and concentration, exact concentrations were not obtainable.

TSE sensitized with crude protein A were agglutinated by all the IgG fractions isolated. *The smallest concentrations capable* of effecting haemagglutination are shown in Table 4, which also shows the minimal concentrations effecting agglutination of mucopeptide. Apparently, the fractions isolated on columns I and II are most effective in both reactions. The observed difference between these two fractions is in both cases only one dilution step and thus not significant.

DISCUSSION

The experiments with the immunoadsorbent columns in conjunction with haemagglutination and the agglutination of mucopeptide clearly showed the presence of at least four different antibody specificities in the anti mucopeptide serum. Consequently, corresponding determinants are present in the mucopeptide preparation. Three of these determinants apparently belong to the peptide subunits. The pentapeptide (P_1 = L-Ala γ-D Glu L-Lys D Ala D Ala) thus seems to have two determinants, one of which is shared by P (L Ala γ D Glu L-Lys D Ala), the other (C terminal D Ala D Ala) by P_3 (Gly L-Ala D Ala D Ala). The glycine bridge (P_4) has one determinant (Tables 2 and 3). This is in accordance with earlier indications (4, 6) and the suggestion that the indirect haemagglutination of TSE sensitized with staphylococcal extracts or preparations containing sensitizing substance is complex (6). It has been found that the TSE sensitizing substance present in extracts or antigen preparations of staphylococci is heterogeneous on a molecular weight basis and that the ratio between different determinants may vary from one preparation to another (*A. Grov* unpublished). The result of inhibition experiments with such systems depends not only on the relative amounts of antibody specificity of the serum but also to some extent on the relative amount of the different active groups present in the sensitizing substance (cfr. 6). Since in the present experiments the tetrapeptide (P) was the most effective inhibitor, the major portion of antibodies in this serum *is most likely directed against the common* determinant of P_1 and P (Table 4).

The seeming contradiction between the present inhibition of indirect haemagglutination and inhibition of the precipitin reaction of mucopeptide in Streptococcus group A variant sera (21) is thus easily explainable. In the latter case it was found that pentapeptide was most effective as an inhibitor and D Ala D Ala as an immunodominant, indicating that antibodies with specificity for D Ala D Ala were dominant in the serum used.

The specificity of the IgG fraction isolated on column III (coupled P_3 = Gly L-Ala D Ala D Ala) apparently reflects the D Ala D Ala structure, which is the only one common to P_3 and P_1. The structure responsible for the common determinant of P_1 and P is obscure. Since the Sepharose was activated by cyanogen bromide, the peptides are *linked* through the terminal amino groups. In the two latter peptides (P and P_2) the α-amino group of Ala as well as the ε amino group of Lys are available for linkage and it is reasonable to believe that both groups are involved. The possible presence of N terminal *L-Ala* revealing an unsubstituted N terminus in the intact mucopeptide has been demonstrated (20) and antibody formation against the N terminal L-Ala may therefore be *elicited*. However, in the precipitin inhibition studies (21) both D Glu and L-Lys, in contrast to L Ala, were found to be of importance, which may suggest that responsibility for the determinant common to P_1 and P_2 lies with D Glu and/or L-Lys. Studies on antigen antibody interactions using synthetic oligopeptides (19) have strongly indicated that *the size* of the

combining sites of antibodies was in all cases such as to accommodate 4 amino acid molecules, and therefore that the antigenic determinant was a tetrapeptide However, an antigen can provoke antibodies against many different determinants present in its molecule, and some of them may overlap The two determinants in P_1 probably overlap, but since no cross reaction was observed between P_2 and P_3 the amino acids in common must be of minor importance to at least one of the determinants

The antibodies isolated on column IV (coupled $P_4 = (Gly)_5$) are probably directed against the COOH terminus of the glycine bridge which is usually blocked (attached to ε NH_2 of Lys) in intact mucopeptide, but the presence of autolytic endopeptidases is frequent among staphylococci (24)

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THE NATURE OF PHOSPHOLIPASE C FROM *ACINETOBACTER CALCOACETICUS*: EFFECTS ON WHOLE RED CELLS AND RED CELL MEMBRANES

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The effect of chelating agents and divalent metal ions on the enzymatic reaction of phospholipase C (haemolysin) of *Acinetobacter calcoaceticus* has been examined Various phospholipids have been tested for their interference with phospholipase C induced haemolysis The effects of the enzyme on red cell membrane phospholipids and red cell membrane adenosine triphosphatase (ATP ase) activities have been examined In accordance with previous investigations, the total enzymatic activity (TA) was found to consist of a basal activity (BA) in the absence of Mg^{2+}, plus an increase in activity induced by the addition of Mg^{2+} (MgA) Treatment of phospholipase C with 5 mM ethylendiaminetetraacetate (EDTA) prior to incubation with substrate, completely inactivated the enzyme Addition of 5 mM Mg^{2+} restored 27 per cent of TA while 0 1 mM Zn^{2+} did not reactivate the enzyme The inhibitory effect of EDTA in the incubation mixture was overcome by Mg^{2+} In the haemolytic reaction, Zn^{2+} inhibited MgA in concentrations that had no detectable effect on BA Micellar phospholipids were shown to have an inhibitory effect on haemolytic activity The enzyme attacked lecithin, phosphatidylethanolamine and sphingomyelin of the red cell membrane, liberating 69 per cent of the membrane bound acid soluble phosphorus Both ouabain sensitive and insensitive ATP ase activities were partly inactivated

Previous investigations (6-9) have shown that ethylendiaminetetraacetate (EDTA) and zinc ions inhibit, and magnesium ions enhance, the haemolytic (phospholipase C) activity of *Acinetobacter calcoaceticus* A basal activity was, however, present when no free divalent metal ions were added to the incubation mixture

A purified phospholipase C preparation was active against various isolated phospholipids (9) Among these were the major phospholipid constituents of human red cell membrane, *i e* lecithin (PC), phosphatidylethanolamine (PE) and sphingomyelin (Sph)

Phospholipase C from other [illegible] example from *Clostridium perfringens* [illegible] and *Bacillus cereus* (4), has been [illegible] hydrolyse phospholipids in red cell [illegible] preparations Phospholipase C [illegible] *fringens* is also capable of [illegible] red cell membrane adeno[illegible] (ATP-ase) activity (19) [illegible] cumulated that phospholipase [illegible] *fringens* (5) and *B cereus* [illegible] metalloenzymes

This paper reports [illegible]

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the role of chelating agents, Zn^{2+} and Mg^{2+} in the enzymatic reaction of phospholipase C from *A. calcoaceticus*. Pure phospholipids have been tested for their interference with phospholipase C induced haemolysis. The action of the enzyme on the phospholipids of red cell ghosts and on red cell ghost ATP-ase activity has also been examined.

MATERIALS AND METHODS

The strain of *A. calcoaceticus* (1318/69), the culture medium, the cultivation technique (9), the assay of phospholipase C (8) and the method for estimation of haemolytic activity (6) have been described in previous reports.

Chemicals

Pure phospholipids and diglyceride were purchased from Koch Light Lab., Colnbrook, Buckinghamshire, England, and were the same as described in a previous paper (9). In addition, lysolecithin (LPC) CHR batch no. 52929 and L-α-glycerylphosphorylcholine (GPC) CHR, batch no. 39793 were used. Bovine albumin (fraction V, from bovine plasma) was obtained from Armour Pharmaceutical Company, Eastbourne, England. Phosphorylcholine chloride, ethylenglycol bis (β aminoethyl-ether) N, N'-tetraacetic acid (EGTA), adenosine 5-triphosphate (Tris ATP) and oualbain were supplied by Sigma Chemical Company, St. Louis, Mo., USA. Ethylendiaminetetraacetate (EDTA) was obtained from E. Merck AG, Darmstadt, West Germany. Other chemicals used were analytical grade.

Enzyme Solutions

1. For experiments on red cell membrane preparations, culture supernatants were purified by dialysis ultrafiltration and salt gradient chromatography on DEAE Sephadex, as previously described (9). The specific activity (H.U./ml/A_{280}) of the freshly prepared solutions ranged between 1950 and 2100. For other purposes culture supernatants were concentrated by ultrafiltration and dialysed against tap water. After this the dialysates were dialysed against 200 volumes of glass distilled water and stored at −20° C until used.

Treatment of Enzyme and Substrate (PC) with EDTA before Incubation

To examine the effect of EDTA on the enzyme the enzyme solution was incubated with 5 mM EDTA (0.05 M tris pH 7.6) overnight at 4° C then dialyzed for 24 h at 4° C against 3 portions of 500 volumes glass-distilled water, and finally incubated with PC. A control, identically treated but without EDTA, was included. To eliminate possible traces of divalent metal ions from the substrate (PC), a suspension (30 mM) obtained by ultrasonic disintegration, was treated twice with 5 mM EDTA (pH 8.3), followed by extraction into an equal volume of chloroform methanol 2:1 (v/v). The extract was washed repeatedly in glass distilled water, evaporated to dryness by N_2 and suspended in 0.2 M tris pH 8.3, with 500 mM NaCl, to a concentration of 30 mM PC. Final concentration in the incubation mixture for assay of phospholipase C was 7.5 mM PC.

Experiments on whole cells. The effect on haemolysis was examined with Zn acetate, EDTA, EGTA, phospholipids, diglyceride and phosphorylcholine dissolved in 0.1 M tris pH 7.6, with 130 mM NaCl. Phospholipids and diglyceride were suspended in the buffer by treatment with a Mullard Ultrasonic Disintegrator at 20 kc/s for 10 min. Final concentrations were as specified in each experiment.

The enzyme solution was serially diluted in two-fold steps in 0.145 M NaCl. To each portion of 0.5 ml was added 0.25 ml of the agents tested and 0.25 ml of a 2 per cent (v/v) human red cell suspension, suspended in the same buffer as the agents. When present, $MgCl_2$ and albumin were added to the red cell suspension, final concentrations being as specified in each experiment. In one experiment (see text) the red cells were washed 5 times in 30 volumes 0.145 M NaCl with 1 mM EDTA followed by 3 washings in 0.145 M NaCl prior to incubation with the enzyme solution. Unless specified, incubations lasted for 20 min at 37° C. Experimental details are described in the figures.

Experiments on Red Cell Membrane Preparations

Preparation of ghosts. For determination of the enzymatic action on membrane phospholipids ghosts were prepared essentially as described by *Parpart* (14). Red cells from 20 ml citrated human whole blood were washed 3 times in 0.145 M NaCl. The cells were then haemolysed in 10 ml distilled water, and the ghosts were sedimented overnight at 4° C in 1500 ml distilled water, saturated with CO_2. The supernatant was then removed, the sediment was collected, centrifuged at 2000 g for 20 min, and then washed 5 times with CO_2-H_2O at 4° C. Finally the ghosts were suspended in 0.05 M tris pH 8.3. The ghost suspension contained 1.2 μM P/ml (examined according to (*Bartlett* (2)), and 1.1 mg protein/ml (examined according to *Lowry et al.* (11)).

The effect of phospholipase C on red cell membrane ATP-ase was examined with a ghost suspension prepared according to *Post et al.* (15). The ghost suspension was made in 5 × 10^{-4} M histidine imidazole buffer pH 7.1 and was found to contain 1.65 mg protein/ml. Freshly prepared ghost suspensions were used.

Examination on the Effect on Membrane Phospholipids

8 ml ghost suspension, 7 ml enzyme solution and 1 ml 80 mM $MgCl_2$ were mixed and incubated at 37° C Immediately after mixing, and after 4 h incubation 7 5 ml of the mixture were precipitated with 1 5 ml 30 per cent (w/v) trichloracetic acid (TCA) Supernatants (S) and precipitates were separated by centrifugation The precipitate was washed 3 times in 5 per cent TCA and then extracted overnight by chloroform methanol (c/m 2 1, v/v) After 2 washings with small volumes of distilled water, the extract was reconstituted to 10 ml by c/m 2 1 (E) Aliquots of S and E were examined on total phosphorus concentration according to *Bartlett* (2)

A sample of 1 ml was withdrawn from the incubation mixture at 1 h incubation, and precipitated with 0 2 ml 30 per cent TCA Determination of total phosphorus concentration in this supernatant served as a control on the course of the enzymatic reaction Controls devoid of enzyme showed constant phosphorus values during the incubation period

9 ml of E were concentrated to 1 ml by evaporation in N_2 and double sets of 100 µl portions were subjected to thin layer chromatography on silica gel plates (20 × 20 cm thickness 0 3 mm, activated for 2 h at 110° C) To identify the phospholipid fractions, pure phospholipids (PC, PE and Sph) were run simultaneously The developing system was chloroform methanol water (65 25 4, v/v/v) Separated lipids were screened by a molybdenum spray (3) and permanently visualized by charring at 110° C after spraying with 9 M H_2SO_4 After visualization, the spots of membrane-derived phospholipids (PC, PE and Sph) were carefully scraped off the plate, and the phosphorus content was estimated after wet ashing in 60 per cent (v/v) perchloric acid at 140° C for 18 h A scraping from the unused part of the plate was used as a blank

Examination of the Effect on Red Cell Membrane ATP ase Activity

Ouabain sensitive and insensitive ATP ase activity of red cell ghosts was measured according to *Post et al* (15), with minor modifications The reaction mixture (2 5 ml) consisted of 0 2 ml ghost suspension, 2 mM $MgCl_2$, 80 mM NaCl, 33 mM KCl 40 mM histidine, 40 mM imidazole (pH 7 1), 2 mM ATP and, when present, 1 mM ouabain

In order to determine whether phospholipase C had an inhibitory effect on ATP ase activity, the mixture was incubated at 37° C for 60 min with 0 75 ml enzyme solution (79 H U of phospholipase C) prior to the addition of ATP and ouabain Then after rapid cooling in ice water, ATP and ouabain were added to a final volume of 2 5 ml Immediately upon the addition of ATP and ouabain, and after 45 min at 37° C, portions of 1 25 ml were precipitated with 1 25 ml 10 per cent TCA, followed by estimation of inorganic phosphorus in the TCA supernatants according to *Rathbun & Betlach* (16) To estimate non inhibited ATP ase, controls that were identically treated, but were devoid of phospholipase C, were included Blanks showed no detectable increase of in organic phosphorus from the ghost suspension A spontaneous release of phosphorus from ATP, corresponding to 7 per cent of total ATP ase activity, was corrected for in the calculations of ATP ase activity

TABLE 1 *Effect of Treatment of Phospholipase C with Ethylendiaminetetraacetate*

Reactivation by divalent metal ions	Enzyme activity (µmol P/ml/h) EDTA treated enzyme	Control
None	0	0 28
0 1 mM Zn^{2+}	0	0 07
5 0 mM Mg^{2+}	0 19	0 71

Experimental details are described in the text

RESULTS

The action of phospholipase C on EDTA-treated PC was investigated with and without 5 mM Mg^{2+} in the incubation mixture Treatment of PC with EDTA prior to incubation with the enzyme had no effect on phospholipase C activity

PC was incubated with phospholipase C treated with 5 mM EDTA prior to incubation (Table 1) When no divalent ions were added to the reaction mixture, the activity was completely inhibited Addition of 0 1 mM Zn^{2+} did not reactive the enzyme, while 5 mM Mg^{2+} restored 27 per cent of the activity Pretreatment of the enzyme with 1 mM EDTA instead of 5 mM EDTA had no significant inhibitory effect

In accordance with previous investigations (6), the total enzymatic activity (TA) was found to consist of a basal activity (BA), present without the addition of Mg^{2+}, plus an increase in activity induced by the addition of Mg^{2+} (MgA) This phenomenon was present

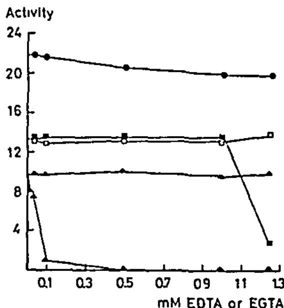


Fig 1 Effect of Chelating Agents on Haemolytic Activity Enzyme solution (20 8 H U./ml) was serially diluted 1 1 in 0 145 M NaCl To portions of 0 5 ml was added 0 25 ml EDTA (●■▲) or EGTA (□△), final concentration being shown in the figure, and 0 25 ml 2 per cent (v/v) red cells suspended in 0 1 M tris pH 7 6 with 130 mM NaCl When present $MgCl_2$ was added to the red cell suspension Final concentrations of Mg^{2+} were 0 mM in △ and ▲ 1 mM in □ and ■, and 5 mM in ● Haemolytic activity was estimated according to the standard method (6)

both in the haemolytic reaction and in the reaction with isolated PC

Washing the red cells with EDTA immediately prior to incubation with enzyme reduced BA to 60 per cent of the control value, *while TA was unaffected, or even increased* slightly In the haemolytic reaction low concentrations of EDTA inhibited BA, while TA was unaffected until the concentration of EDTA exceeded that of Mg^{2+} (Fig 1) EGTA did not show any inhibitory effects Similar results were found with isolated PC as a substrate In the haemolytic reaction BA was unaffected by concentrations of Zn^{+} (up to 0 1 mM) that inhibited MgA (Fig 2) The inhibition was more pronounced at 1 mM Mg^{2+} than at 5 mM Mg^{2}

PC showed a marked inhibitory effect on haemolysis Under the conditions imposed 50 per cent inhibition occurred with less than 5 μM PC (Fig 3) PE and LPC exhibited moderate inhibitory effects, while Sph and phosphatidylserine (PS) were weak inhibitors GPC did not inhibit significantly, phospho*rylcholine showed no inhibition up to a con*centration of 1 25 mM, while diglyceride inhibited approximately to the same extent as Sph and PS

At concentrations of 50 μM or higher PC itself excerted a 'hot cold' haemolytic effect (haemolysis after cooling a mixture of PC and red cells) The other phospholipids including LPC were not haemolytic in the concentrations used Albumin (10 mg/ml), which has previously been shown to accelerate phospholipase C activity (9), interfered neither with the inhibition of haemolysis by

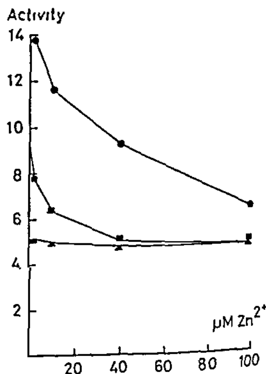


Fig 2 Effect of Zinc Ions on Haemolytic Activity The experimental conditions were the same as in Fig 1, expect that the activity of the enzyme solution was 13 8 H U./ml and that *Zn acetate* was added instead of chelating agents Final concentrations of Zn^{+} are seen in the figure final concentrations of Mg^{2+} were 0 mM (▲) 1 mM (■) and 5 mM (●)

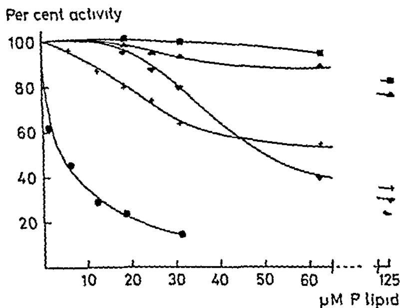


Fig 3 Effect of Various Phospholipids on Haemolytic Activity The experimental conditions were the same as in Fig 1 expect that phospholipids were used instead of chelating agents Activity was calculated in per cent of the haemolytic activity in the enzyme solution ● lecithin ▼ lysolecithin, ■ sphingomyelin + phosphatidylethanolamine ▲ phosphatidylserine

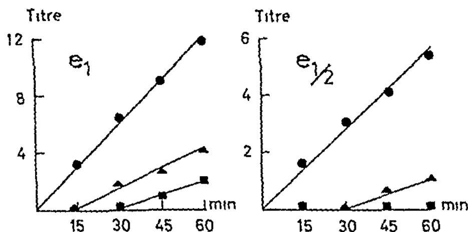


Fig 4 Effect of Lecithin on the Course of the Haemolytic Reaction Incubation and estimation of activity were the same as in Fig 3 Two concentrations of enzyme were tested $e_{1/2} = e_1$ (11 8 H U/ml) diluted 1 1 in distilled water Samples were analysed at 15 30 45 and 60 min ■ 12 5 μM PC ▲ 3 13 μM PC ● without PC

PC nor with the non specific hot-cold haemolytic effect of PC

The inhibitory effect of PC on haemolysis was examined at lower enzyme concentrations (Fig 4) PC produced a lag period in the haemolytic reaction and also a decrease in haemolytic activity after the lag period The lag period increased with increasing concentration of PC, and also with decreasing enzyme concentration

TABLE 2 *Effect of Phospholipase C on Phospholipids in Red Cell Ghosts*

Phospholipid	Hydrolysis per cent*	Incubation period
Total phospholipid (as P)	65 ± 3	1 h
Total phospholipid (as P)	69 ± 2	4 h
Lecithin (PC)	74 5 ± 5	4 h
Phosphatidylethanolamine (PE)	77 ± 3	4 h
Sphingomyelin (Sph)	72 5 ± 2	4 h

* Mean value of two sets

TABLE 3 *Effect of Phospholipase C on Red Cell Membrane ATP-ase*

	Inactivation %
Total ATP ase	66
Ouabain sensitive ATP ase (32 per cent of total ATP ase)	43
Ouabain insensitive ATP ase 68 per cent of total ATP ase)	76

Phospholipase C from *A calcoaceticus* acted upon membrane bound PC PE and Sph, releasing 69 per cent of the total membrane phosphorus in 4 h (Table 2) The major part of this (95 per cent) was released during the first hour of incubation

Both the ouabain sensitive and -insensitive ATP-ase of red cell ghosts were partly inhibited by the action of phospholipase C (Table 3)

DISCUSSION

The inactivation of the enzyme by treatment with 5 mM EDTA and the partial reactivation by Mg^{2+} strongly indicate that a divalent metal ion is a cofactor of the enzyme The cofactor was not removed after dialysis against water, inert buffers or 1 mM EDTA and must therefore be firmly attached to the enzyme molecule When present in a reaction mixture devoid of divalent metal ions EDTA showed a marked inhibitory effect while in the presence of Mg^{2+}, a significant inhibition of activity occurred only if the concentration of EDTA exceeded that of Mg^{2+}

Phospholipase C from *Cl perfringes* and *B cereus* are thought to be zinc metalloenzymes The enzyme in question was not reactivated by Zn^{2+} On the contrary, Zn^{2+} (and Ca^{2+}) have previously been shown to have an inhibitory effect on the phospholipase C activity when present in the reaction mixture (9) EGTA, which chelates Zn^{2+} and Ca^{2+}, but Mg^{2+} only very weakly, did not have an inhibitory effect on the enzymatic reaction These observations make it probable that phospholipase C from *A calcoaceticus* is a metalloenzyme, requiring Mg^{2+}, or some other divalent metal ion distinct from Zn^{2+} or Ca The cofactor is essential to the basal activity (BA) of the enzyme Apart from this free divalent metal ions like Mg^{2+} or Mn^{2+} accelerate the enzymatic activity possibly due to an effect on the surface charge of the substrate (1), and protect the enzyme from inactivation by EDTA

Some differences between the haemolytic reaction and the reaction with isolated PC were noted Washing of the red cells with EDTA prior to incubation with enzyme decreased BA, while washing of *PC* with *EDTA* had no effect In the haemolytic reaction 0 1 mM Zn^{2+} counteracted the effect of free Mg^{2+} (MgA), while BA was unaffected However, in the reaction with isolated PC 0 1 mM Zn^{2+} decreased BA (Table 1 see also (9)) Previous investigations have also shown that while 5 mM Ca^{2+} has little influence on haemolytic activity (6), it has an inhibitory effect on the reaction with isolated PC (9) These discrepancies are not easily interpreted but could indicate that divalent metal ions or other charged groups attached to the surface of the red cell membrane participate to some extent in the enzymatic reaction resulting in *haemolysis*

A recent report (9) demonstrated that purified phospholipase C from *A calcoaceticus* attacks micellar PC PE, PS and Sph The inhibitory effect of micellar phospholipids on haemolysis was most pronounced with *PC* while Sph and PS showed remarkably weak inhibitory effects From the experiment presented in Fig 4 the inhibitory effect of *PC*

is most probably due to substrate competition

The reaction products of phospholipase C action on PC, diglyceride and phosphorylcholine, showed very faint or no, inhibitory effects on haemolysis, indicating that the reaction product type of inhibition was not a characteristic feature of the enzymatic reaction

The enzyme attacked PC, PE and Sph of the red cell membrane preparation A complete hydrolysis was, however, not obtained This is in agreement with the results of *Lenard* & *Singer* (10), working with the clostridial enzyme, and with those of *Glaser et al* (4), using both clostridial and *B cereus* phospholipase C With clostridial phospholipase C, *Roelofsen et al* (18) obtained a complete hydrolysis of PC, PE and PS, but no significant hydrolysis of Sph

Inhibition of red cell membrane ATP ase by clostridial phospholipase C was described by *Schatzmann* (19) The inhibitory effect was thought to be due to degradation of membrane phospholipids serving as prosthetic groups for the ATP ase Further evidence for this has been presented by, for example *Roelofsen et al*, using *B cereus* phospholipase C (17) Both authors found that both types of the red cell membrane ATP ase were affected Phospholipase C from *A calcoaceticus* has a similar effect, the ouabain-insensitive ATP ase being inactivated to a greater extent than the ouabain sensitive ATP ase

Phospholipase C from *A calcoaceticus* shows many features in common with other phospholipase C enzymes However, some of its properties indicate specific structural differences from other known enzymes of this type Its haemolytic activity is evidently due to degradation of red cell membrane phospholipids principally PC leading to structural and functional disorders and finally to lysis of the red cell

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HAEMOLYTIC ACTIVITY OF VARIOUS STRAINS OF *ACINETOBACTER*

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Extracellular and intracellular haemolysin (phospholipase C) of *Acinetobacter calcoaceticus* have been examined during bacterial growth in a chemically defined medium The haemolytic activities of various strains of *Acinetobacter*, grown in broth media have been compared Intracellular haemolytic activity was detected only at the early stage of growth Release of haemolysin from the cells started at this point and reached its maximum at the end of the logarithmic phase of growth In *A calcoaceticus* (anitratus), 6 out of 17 strains were haemolytic to human red cells and in *A lwoffi* 2 out of 13 strains In broth culture filtrates two distinct types of haemolysis occurred In some strains haemolysis increased after cooling (hot cold effect), while in others a regular direct haemolysis occurred at 37° C with no detectable increase in lysis after cooling Both types of activity were inactivated by heating and by ethylendiaminetetraacetate and activated by Mg^{2} and albumin All haemolytic filtrates examined released acid soluble phosphorus from lecithin The two types of haemolytic activities showed immunological differences

Studies on the haemolytic activity (phospholipase C, EC 3 1 4 3) of a local strain (1318/69) of *Acinetobacter calcoaceticus* have been published in recent reports (7 11 *)

On blood agar, visible growth and haemolysis occurred simultaneously In liquid media, the release of haemolysin started early in the logarithmic growth phase, reached a maximum at the end of this phase and decreased as the culture entered the stationary and autolytic phases of growth (7, 9) This indicated that the haemolysin was excreted by actively growing cells, rather than being released from cells which were undergoing death and lysis Evidence of true extracellularity of the haemolysin of *A calcoaceticus* is presented in this report

Haemolysis on blood agar is a common finding in the strains of *Acinetobacter* A comparative investigation of the haemolytic activity of strain 1318/69 and some other strains of Acinetobacter has been carried out

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* Recently it has come to the author's attention that *Andreoni et al* (15) described the presence of a phospholipase in strains of *Moraxella glucidolytica* The properties of the enzyme were not described in detail It seems however, likely that the strains belong to the *genus Acinetobacter* and it is possible that the enzyme is the same as that discussed by the author

MATERIALS AND METHODS

Determination of Haemolytic Activity

Unless specified, this was performed according to the standard method, published earlier (7) One haemolytic unit (H U) was defined as the amount

of haemolysin that would produce 50 per cent haemolysis of 1 ml 1 per cent human red cell suspension by the method used

Estimation of Intracellular and Extracellular Haemolysin

The culture medium (a chemically defined low molecular weight medium) has been described earlier (9) Three ml of a 24 h culture of strain 1318/69 was inoculated into 300 ml of the medium and incubated at 28° C for 120 h in slowly agitated Erlenmeyer flasks Every 24 h a portion of 40 ml was removed and the growth rate was estimated turbidimetrically (A_{620}) Cells and supernatant (S) were separated by centrifuging in a Servall Superspeed Centrifuge (9000 g 50 min, 4° C) The cells were washed 3 times in 40 ml 0 145 M phosphate buffered NaCl pH 7 2 (PBS) After washing the pellet was suspended in 5 ml PBS and treated in a Mullard Ultrasonic Disintegrator for 15 min at 20 kc/s To minimize temperature rises the tubes were kept in ice water during the treatment Bacterial counts showed that 91 ± 3 per cent of the bacteria were destroyed by this treatment In Gram stained preparations, the bulk of the bacteria was shown to be converted into debris and amorphous masses. To estimate the degree of inactivation of the haemolysin by the ultrasonic treatment 5 ml of the supernatant (S) was treated in the same way as the cell suspensions. The haemolytic activity was reduced by 32 ± 5 per cent

The ultrasonic cell lysate (L) was centrifuged to remove debris S and L were kept at −20° C and examined simultaneously

Protein was estimated as the absorbance at 280 nm Haemolytic activity in S was expressed as H U./ml In L the haemolytic activity was estimated as H U/ml/cell mass unit (turbidity of the culture)

Investigation of the Haemolytic Activity in Various Strains

Strains of *A calcoaceticus* and *A lwoffi* were obtained from (1) Czechoslovak Collection of Microorganisms J E Purkyne University Brno Czechoslovakia *A calcoaceticus* CCM 1967 1989 2259 2265 2315 2355 2357 2358 5593 5574 5594 5596 5721 and 5567 *A lwoffi* CCM 1976 2065

in the study

Media

The lyophilized organisms were cultured at 37° C in a broth medium (Oxoid) consisting of Proteose Peptone 15 g/l Liver Digest 2 5 g/l Yeast Extract 5 g/l, NaCl 5 g/l, pH 7 2 This medium was also used for production of culture filtrates from haemolytic strains Haemolysis on blood agar (6 per cent human blood) was used for the identification of haemolytic strains Acid production from carbohydrates was examined in a broth medium containing 1 per cent (w/v) carbohydrate (lactose also 10 per cent) Sensitivity to antibiotics was examined according to *Ericsson* (3)

Preparation of Haemolytic Culture Filtrates

Broth medium was inoculated with 1/100 volume of a 24 h broth culture of the strains examined and incubated aerobically in Roux bottles at 25° C. Growth was estimated turbidimetrically every 24 h Culture filtrates were obtained at the same intervals by centrifugation and filtration as previously described (9) and examined on haemolytic activity Filtrates from cultures grown for 48 h were dialysed against tap water and were kept at −20° C for further examination

Influence of Mg^{2+}, Albumin and Ethylendiaminetetraacetate (EDTA)

Bovine albumin (fraction V from bovine plasma) was obtained from Armour Pharm Company Eastbourne, England and EDTA (disodium salt) from E Merck AG Darmstadt Western Germany The reagents were added to the red cell suspension and incubated with haemolysin according to the standard method Final concentrations are seen in Table 1

Hot-cold Effect

Hot-cold effect was present when the titre of 100 per cent haemolysis increased by at least one dilution step after cooling of the tubes for 10 min in ice-water

Assay of Phospholipase C

This was performed by estimating the rate of release of acid soluble phosphorus from lecithin ((PC) L-α lecithin ex egg CHR batch no H 3625 Koch Light Laboratories Colnbrook Buckinghamshire England) The experimental conditions have been described previously (9, 11)

Immunization and Estimation of Antihaemolytic Activity

Crude culture filtrate from strain 1318/69 was prepared from a chemically defined medium as previously described (11) After dialysis against 200 volumes of 0 145 M NaCl the filtrate was concentrated to 120 H U./ml in an Amicon Ultrafiltration Cell using a Diaflo Membrane (UM 20

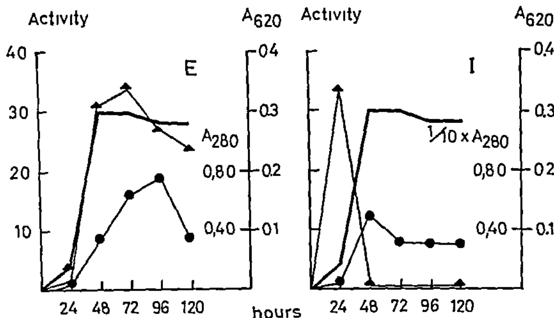


Fig 1 Growth Curve and Haemolytic Activity E Extracellular pattern I Intracellular pattern —— Turbidity of the culture (A_{620}) ▲ Haemolytic activity (in E H U /ml in I H U /ml/cell mass unit) ● protein (in E A_{280} in I 1/10 × A_{280}) See text for experimental details

E, 3 kp/cm² N_2) After collection of preimmune sera, portions of 0 5 ml concentrate were injected intravenously into two six months old rabbits twice weekly for 3 weeks Immune sera were collected 10 days after the last injection Determination of antihaemolytic activity was carried out by twofold serial dilution of the sera in 0 145 M NaCl so that 50 µl remained in each tube Culture filtrates from the strains examined were diluted in 0 145 M NaCl to 6 5 H U /ml and portions of 50 µl were added to each tube After incubation at room temperature for 30 min, 100 µl 1 per cent human red cell suspension was added and the tubes were incubated for 45 min at 37° C The reaction was stopped by cooling the tubes in ice water, followed by addition of 0 4 ml 0 145 M NaCl and centrifuging of the tubes to sediment intact red cells The antihaemolytic titre was calculated as the reciprocal value of the lowest serum dilution to give 100 per cent haemolysis by the method used

RESULTS

Intracellular and Extracellular Haemolysin

The turbidity of the culture reached its maximum between 48 and 72 h (Fig 1) Extracellular haemolytic activity showed maximum values at the end of the logarithmic growth phase, and earlier than extracellular proteins The extracellular haemolysin-protein ratio (specific activity, H U /ml/A_{280}), showed the highest values at 24 h (24 h 350, 48 h 88, 72 h 55, 96 h 39, 120 h 66) Significant amounts of intracellular haemolysin were detected only at the start of the logarithmic phase of growth (24 h)

Examination of Various Strains

The following strains showed haemolysis on blood agar *A calcoaceticus* CCM 1989, CCM 2358, CCM 5567, CCM 1967, CCM 2357 and 1318/69 *A lwoffi* CCM 1976 and CCM 2359 When incubated in broth media all these strains yielded haemolytic culture filtrates Culture filtrates from strains CCM 1989, CCM 2358, CCM 5567, CCM 1967 and CCM 2359 showed hot cold effect (H C strains), while culture filtrates from strains CCM 1976, CCM 2357 and 1318/69 showed a regular, direct haemolysis with no detectable increase in lysis after cooling (D strains)

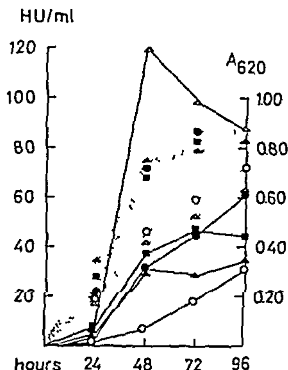


Fig 2 Growth Curve and Haemolytic Activity in Various Strains of Acinetobacter Turbidity of the cultures (A_{620}) —— Haemolytic activity (H U/ml) △ CCM 2358 ○ CCM 5567 ■ CCM 1976 ▲ CCM 2357 ● 1318/69 See text for experimental details

The H C strains CCM 2358 and CCM 5567 and the three D strains were subjected to further investigations

Biochemical and Cultural Properties

All strains were oxidase and indol negative The strains of *A calcoaceticus* produced acid from glucose xylose arabinose galactose and from 10 per cent lactose while with 1 per cent lactose and various other carbohydrates acid production was not detectable The strain of *A lwoffi* (CCM 1976) did not produce acid from any of the carbohydrates

All strains were sensitive to streptomycin tetracyclin and gentamycin but showed a varying degree of sensitivity to several other antibiotics tested

In contrast to the D strains the H C strains failed to grow on a citrate medium and were markedly sensitive to chloramphenicol In a broth medium the H C strains showed a slower growth rate than the D strains (Fig 2)

Haemolytic Activity

The ability to produce haemolytic culture filtrates varied considerably in different strains (Fig 2) The haemolysins were not dialysable The haemolytic activity was completely destroyed after heating the filtrates for 5 min at 56° C (Table 1) The table also shows that the haemolytic activity of all strains was inactivated by EDTA and activated to approximately the same extent by Mg⁺ and albumin Haemolytic culture filtrates from all strains released acid soluble phosphorus from lecithin The H C strains showed a higher ratio between haemolytic activity and activity towards isolated PC than the D strains

Antisera against 1318/69 haemolysin inhibited the haemolytic activity of the D strain filtrates up to titres of 1024 or higher (Table 2) With respect to the filtrates of the H C strains, the D strain antisera showed only a slight increase in antihaemolytic activity in comparison to the preimmune sera These patterns were present both in the hot and the cold phases of incubation

DISCUSSION

The results indicate that the haemolysin (phospholipase C) of strain 1318/69 is an exoenzyme

The haemolytic activity of the strains examined showed many features in common The release of haemolysin started early in the logarithmic growth phase all haemolysins were rapidly inactivated at 56° C were stimulated by Mg and albumin and inhibited by EDTA Evidence of phospholipase C activity was found in all haemolytic culture filtrates and the activity towards isolated PC roughly parallelled the haemolytic activity These findings are in agreement with previous investigations of the haemolytic activity of strain 1318/69 (7 12), and indicate that the haemolytic activity of all strains was en

TABLE 1 *Properties of Haemolytic Activity from Various Strains of Acinetobacter*

rain	Per cent haemolytic activity Control	Heating 56° C for 5 min	EDTA 1 mM	Mg² 5 mM	Albumin 10 mg/ml 5 mM Mg²	Haemolytic activity (H) H U/ml	Activity towards isolated PC (P) µmol P/ml/h	H/
CM 1976	100	0	0	223	267	37 2 D *	0 425	8
CM 2357	100	0	0	212	274	8 7 D	0 083	10
318/69	100	0	0	216	330	9 1 D	0 083	10
CM 2358	100	0	0	167	311	37 5 H C ***	0 201	18
CM 5567	100	0	0	254	329	4 2 H C	0 028	15

* PC lecithin
** direct haemolysis
* increase in haemolytic activity after cooling (hot cold effect)

zymatic in nature and due to phospholipase activity

Some characteristic differences between the strains were, however, found The haemolytic activity of 5 strains showed hot cold effect (H C strains), while in 3 strains only a direct haemolytic reaction was observed (D strains) Some differences in growth characteristics between the two types of strains were also found H C strains showed a somewhat higher ratio than D strains between haemolytic activity and activity towards isolated PC and H C strain haemolysin was only slightly inhibited by antisera against D strain haemolysin

Phospholipids have been shown to inhibit the haemolytic activity of strain 1318/69 (12) Phospholipid containing lipoproteins of the rabbit sera would thus be expected to have an inhibitory effect on the haemolytic activity The preimmune titres (8 32) were most probably due to this mechanism rather than to preimmune antibodies.

A full explanation for the serological differences between the haemolysins of the H C strains and the D strains cannot be given especially since the haemolysins were not purified The marked serological difference between the haemolysins of the two types of strains which was present both in the hot and the cold phases of incubation makes it probable that the two types of haemolytic activities reside in structurally distinct molecules The slight inhibitory effect of D strain antisera on H C strain haemolysin may be due to antibodies This might be explained by a serological cross reaction due to structural analogism or by the presence of small amounts of D strain haemolysin in the haemolytic filtrates from the H C strains

A serological study of the haemolytic activity of various strains of *Acinetobacter* (*Alcaligenes haemolysans*) was published by *Henriksen* (5), employing rabbit erythrocytes and sera

In his experiments normal rabbit serum was not anti haemolytic, and significant serological differences between the various strains were not found

Haemolysis is not a ubiquitous feature of the strains of *Acinetobacter* (4 13) In accor

TABLE 2 *Antihaemolytic Titres in Sera of Rabbits Immunized with Haemolytic Culture Filtrate from Strain 1318/69*

Strain	Rabbit no	Preimmune titre	Immune titre
CCM 1976	1	16	>1024
	2	16	1024
CCM 2357	1	16	>1024
	2	8	>1024
1318/69	1	16	>1024
	2	16	>1024
CCM 2358	1	32	64
	2	16	64
CCM 5567	1	16	128
	2	16	64

More than 90 per cent haemolysis

dance with this only 8 out of 30 strains showed haemolysis on blood agar It is noteworthy that no differences were found between the haemolytic activity of a strain of *A lwoffi* (CCM 1976) and those of the strains CCM 2357 and 1318/69 of *A calcoaceticus*

The taxonomical problems of the oxidase-negative species of the *Moraxella* group (genus *Acinetobacter*) have not yet been solved (1, 6) One of the commonly used criteria for the differentiation between *A calcoaceticus* and *lwoffi*, which was also substantiated in this study, is the lower ability of the latter to produce acid from carbohydrates

Traditionally, the bacterial haemolysins have been classified as toxins Physiological extracellularity is a common feature of the toxins of Gram positive bacteria but is uncommon in the toxins of Gram negative organisms (14) The haemolysins of strain 1318/69 attacks leucocytes (10) and inhibits the red cell membrane adenosine triphosphatase (12) The *in vivo* effects of the haemolysin have, however, not been evaluated due to the lack of sufficiently pure, stable and active preparations Nevertheless, it seems justifiable to classify the haemolysin of *A calcoaceticus* as a cytolytic toxin according to the definition of *Bernheimer* (2)

Further serological and enzymatic studies would help to clarify the relationship between the two types of haemolytic activities observed in genus *Acinetobacter*

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LOCALIZATION OF HOST ANTIGENS IN THE EGG-GROWN INFLUENZA VIRUS PARTICLE

I The Forssman antigen not exposed at the surface of A/PR/8/34 (H0N1)

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Egg grown influenza virus A/PR/8/34 (H0N1) was shown to lose the ability to absorb antibodies to the Forssman antigen as the virus purification procedure became more extensive The haemagglutination caused by non infectious HA material, density $\leq$ 1 14 g/cm³, separated from intact virions by centrifuging to equilibrium in a CsCl gradient, was inhibited by anti Forssman serum No HI effect towards intact virions was obtained with this serum

Egg-grown influenza virus has been reported to bind antibodies to mononucleosis-, Forssman- (F-) and blood group antigens (1, 2, 9, 10) Similar findings concerning the blood group B antigen have been made with parainfluenza virus grown in Rhesus monkey kidney cells (8) On the other hand, potent anti-F sera show no inhibitory effect on egg grown influenza virus haemagglutination (7) in contrast to antisera against the keratosulphate like host antigen (6) Furthermore, immunization of rabbits with purified influenza preparations did not give rise to anti F antibodies (7) The latter findings indicate that the F-antigen, if present, must constitute a very small part of the virus particle

The present investigation aims to purify influenza virus with special regard to F-positive contaminants, and to study the F-activity of this preparation

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MATERIALS AND METHODS

Virus

The A/PR/8/34 (H0N1) strain of influenza virus was grown in the allantoic cavity of 10 day chicken embryos. The strain is given the shorthand notation A_0/PR8

Antisera

Serum from a patient suffering from infectious mononucleosis was tested in HL reactions (see below) with bovine erythrocytes and guinea pig serum

All other sera were raised in rabbits Anti F sera (R 89 and R 745) were produced by intravenous injection of chicken erythrocytes The lytic activity of these sera against sheep erythrocytes could be removed by absorption with guinea pig kidney homogenate, but remained constant after absorption with bovine erythrocytes, thus indicating a specific anti F serum Anti influenza B/Lee serum (R 86) was produced by intravenous injections of allantoically grown influenza B/Lee virus, and the anti-A_0/PR8 serum (R 75) by subcutaneous injections of allantoically grown A_0/PR8 virus in Freund's incomplete adjuvans.

Serological Tests

Virus *haemagglutination* (HA) and *haemagglutination inhibition* (HI) tests were performed in

dance with this only 8 out of 30 strains showed haemolysis on blood agar It is noteworthy that no differences were found between the haemolytic activity of a strain of *A lwoffi* (CCM 1976) and those of the strains CCM 2357 and 1318/69 of *A calcoaceticus*

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Traditionally, the bacterial haemolysins have been classified as toxins Physiological extracellularity is a common feature of the toxins of Gram positive bacteria, but is uncommon in the toxins of Gram negative organisms (14) The haemolysins of strain 1318/69 attacks leucocytes (10) and inhibits the red cell membrane adenosine triphosphatase (12) The *in vivo* effects of the haemolysin have, however not been evaluated due to the lack of sufficiently pure stable and active preparations Nevertheless it seems justifiable to classify the haemolysin of *A calcoaceticus* as a cytolytic toxin according to the definition of *Bernheimer* (2)

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Electron Microscopy

Virus preparations were negatively stained with 2 per cent (w/v) phosphotungstic acid (E Merck) adjusted to neutrality by N KOH and then placed on Formvar and carbon coated 400 mesh copper grids before examination in a Philips EM 300 The accelerating voltage was 80 kV and instrumental magnification 90,000 ×

EXPERIMENTS AND RESULTS

Purification of Virus

The following successive steps were included

1st step The virus-containing allantoic fluid was clarified by centrifugation at 3,000 × *g* for 15 min

2nd step The virus suspension was concentrated and purified by adsorption to and elution from human O erythrocytes The eluate is called *O(-eluate)*

3rd step Two successive differential centrifugation (DC) steps were carried out To avoid damage of virus by tight packing in the pellet on high speed centrifugation, a cushion of 50 per cent (w/v) sucrose in PBS was layered under the virus suspension The virus was recovered from the interface and the sucrose layer and thereafter dialysed against PBS overnight in the cold The cen

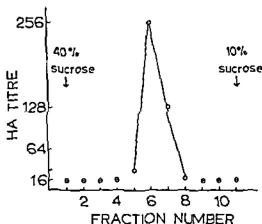


Fig 1 Velocity centrifugation of crude (O/DC) virus in a preformed linear 10-40 per cent sucrose gradient Fraction volume 0 4 ml Codes ⊖ Titre less than , O O-eluate DC Differential centrifugations

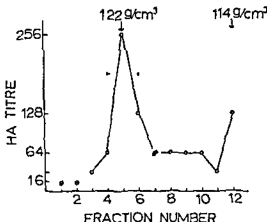


Fig 2 Isopycnic banding of partly purified (O/DC/VC) virus in a CsCl gradient The broken horizontal line represents the pooled fractions called O/DC/VC/IB Fraction 12 is called the IB light fraction Codes VC Velocity centrifugation IB Isopycnic banding Others See legend to Fig 1

trifugation procedure was as follows The O eluate was centrifuged at 27,000 × *g* for 2 h as described above After dialysing away the sucrose from the concentrated virus preparation, this was clarified by centrifugation at 3,000 × *g* for 15 min The supernatant was then once more subjected to the same alternating high- and low speed centrifugations The final preparation is called *O/DC*

4th step Velocity centrifugation (VC) in a sucrose gradient was performed as described in 'Materials and Methods' The pooled and dialysed HA material is called *O/DC/VC* (Fig 1)

5th step Isopycnic banding (IB) in a CsCl gradient was done as described in "Materials and Methods" Influenza virus banded at a density of 1 22 g/cm³ Some HA-active material with a density 1 14 g/cm³ (Fig 2) was recovered on top of the gradient The virus preparation with a density of 1 22 g/cm³, called *O/DC/IB* or *O/DC/VC/IB*, was infectious, and normal virus particles without visible extraneous material were revealed in the electron microscope The HA-active IB-light fraction recovered from the top of the gradient was non infectious, and viral struc-

tures were not seen in the electron microscope

HI tests with Separated IgM and IgG Anti F Antibodies

Anti F antibodies have been shown to lack inhibitory effect against virus mediated haemagglutination (7) In most antisera against the F-antigen IgM is the predominant anti F antibody The possibility exists that IgG antibodies, owing to their smaller size, might be able to bind to antigenic sites on the viral surface i e between the spikes, not accessible to IgM Therefore, the concentrated IgM and IgG preparations were examined for HI activity against different virus preparations Antisera against the A_0/PR8 antigens and against the host antigen were included as positive controls The results are given in Table 1 The table shows that anti F whole serum and concentrated IgM and IgG fractions exhibited no HI effect against the virus preparations The inhibition of the HA active material in the IB-light fraction is of special interest The buoyant density might indicate membrane fractions possibly fragmented virus envelopes or transformed host membranes Antibodies to B/Lee virus show HI effect against A_0/PR8 due to antibodies to the common keratosulphate like host antigen

TABLE 1 *HI Activity of Different Antisera against Influenza A_0/PR8 Preparations*

Antisera	Virus preparation*	HI titre
anti B/Lee	O eluate	256
	O/DC/IB	256
anti A_0/PR8	O eluate	512
	O/DC/IB	512
anti Forssman	O eluate	<4
(R 745)	O/DC/IB	<4
	IB light fraction*	8
R 745	O eluate	<4
separated IgM	O/DC/IB	<4
R 745	O eluate	<4
separated IgG	O/DC/IB	<4

* See legends to Fig 1 and 2

TABLE 2 *Absorption of Anti B/Lee and Anti F Serum with Human O erythrocytes Sensitized with Different A_0/PR8 Preparations*

Absorption with erythrocytes sensitized with	Titre HI	Titre HI	Activity remaining %
PBS (Serum control)*	256		100
O-eluate	64		25
O/DC/VC/IB	64		25
PBS (Serum control)**		65 536	100
O DC		2 048	3
O/DC/VC		32 768	50
O/DC/VC/IB		65 536	100
O/DC/IB		65 536	100

* Serum anti B/Lee diluted 1 64 (i e 4 HI doses/0 25 ml) before absorption

** Serum anti F (R 89) diluted 1 2 048 (i e 32 HI doses/0 25 ml)

Absorption Experiments with Different Virus Preparations

The lack of HI activity in anti F sera does not rule out the possibility that F determinants are exposed on the viral surface because an HI requires complete mechanical blocking of the haemagglutinin spikes Antibodies to the exposed neuraminidase antigens for example do not always inhibit virus mediated haemagglutination Therefore absorption experiments were required

To minimize interference by extraneous host material in the tests erythrocytes were coated with purified virus and thereafter incubated with immune serum In this way only substances capable of binding to erythrocytes were included in the absorption experiments

Table 2 shows that A_0/PR8 influenza virus bound to human O erythrocytes absorbed HI antibodies from an anti B/Lee serum (R 86) This is as mentioned earlier due to removal of antibodies to the host antigen Both the crude O/DC and the purified O/DC/VC/IB preparations effectively removed these antibodies Virtually the same results were obtained with virus attached to guinea pig erythrocytes (not included in the table)

Table 2 also shows that as the virus puri

fication proceeds, the ability to reduce the haemolytic activity of the anti F serum is gradually lost It is obvious that the velocity centrifugation (VC) step is not essential in this respect when a final isopycnic banding (IB) in CsCl is performed

A homogenate of non infected chorioallantoic membranes and allantoic fluid from 10 day old embryonated eggs was subjected to the same purification procedure as the infected allantoic fluid for preparation of the O eluate Human O erythrocytes were sensitized with this material and an absorption experiment performed with anti F serum (R 89) in the usual manner No reduction of HL activity was recorded indicating that the absorbing capacity of the crude virus preparation is not due to contaminating F positive fragments from non infected host material

Another control was also included Virus sensitized human O erythrocytes were examined for the capacity of reducing the haemolytic activity of a human mononucleosis serum against bovine erythrocytes No reduction of the HL-titre was obtained This indicate that the virus sensitized erythrocytes neither exhibit anti complementary effect nor absorb IgM antibodies unspecifically

An additional experiment was done with a higher dose of virus and only 4 lytic doses of 0 25 ml of the antiserum 2 5 ml of virus, O/DC/IB with HA titre 256, was incubated with 2 5 ml anti F serum (R 745), diluted

TABLE 3 *Absorption of Immunesera with Purified (O/DC/IB)* A_0/PR8 Virus in Suspension*

Serum	Titre before/after absorption**		Activity remaining %
anti F (R 745)	(HL)	65 536/65 536	100
anti B/Lee	(HI)	256/64	25
anti A_0/PR8	(HI)	512/64	12

* See legends to Fig 1 and 2

** 2 5 ml of virus HA titer 256 was added to the same volume of the respective sera diluted to contain 4 HL doses/0 25 ml (1 16 384), 8 HI doses/0 25 ml of anti B/Lee (1 32) and 16 HI doses/0 25 ml of anti A_0/PR8 (1 32)

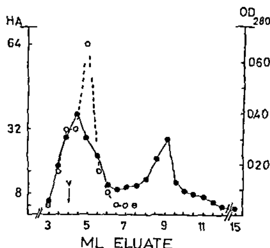


Fig 3 Gel filtration of crude (O/DC) virus on a 100 × 10 mm Sepharose 6B column equilibrated with PBS The fractions of 0 5 ml each were eluted with the same buffer V Void volume ● Optical density at 280 nm ○ HA titres ⊖ Titre less than

1 16 384 in PBS After 30 min at 37° C the mixture was cooled and ultracentrifuged at 114 000 × g for 30 min Also anti B/Lee (R 86) and anti A_0/PR8 (R 75) sera were tested, using 8 to 16 HI doses/0 25 ml The results are given in Table 3, which shows that antibodies to the A_0/PR8 antigens and to the host antigen (in the anti B/Lee serum) are readily absorbed In contrast, no reduction in the lytic activity of the anti F serum was recorded

Separation of F activity from Virus Preparations by Gel Filtration

By applying the crude virus suspension (O/DC) on a Sepharose 6 B column equilibrated with an anti F serum (R 89) and eluting the fractions with the same serum a rough estimate of the size of the F active components can be made The experimental set up is described in Materials and Methods The results are given in Figs 3 and 4

Figs 3 and 4 show that the F-positive material is recovered in the void volume together with the main HA peak The somewhat slow regeneration of the HL-titre is due to the antibody deficiency in the fractions eluted

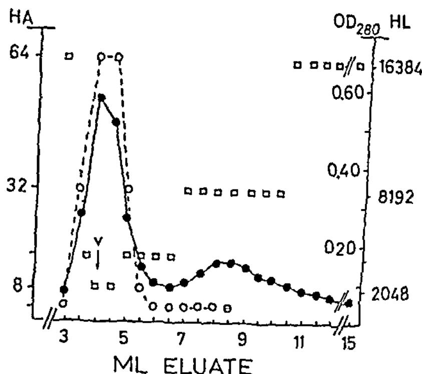


Fig 4 Gel filtrat on of crude (O/DC) virus on a 100 × 10 mm Sepl arose 6B column equ l brated with an anti F serum (R 89) (see Methods) Tl e fractions of 0 5 ml each were el ited vith the same serum Codes □ HL-titres. Otl er See legend to Fig 3

after the HA peak When comparing the protein peaks in void volume (Figs 3 and 4) it is clear that serum proteins (i e antibodies) are bound Purified virus (O/DC/IB), applied on the same column as a control did not reduce the HL activity in the serum eluted fractions The protein concentration in the vo d volume did not exceed the serum background (not included in the figures) When the IB light fraction (fraction 12 in Fig 2) was applied a reduction in HL activity was observed which was similar to that of the O/DC material The results of these experiments are thus in complete accordance with those of the absorption tests

DISCUSSION

The HI tests with anti F sera and influenza virus preparations presented here and earlier (7), show that if F determinants are exposed on the viral surface they must be widely spaced since no inhibition of virus mediated HA was obtained Theoretically IgG antibodies may reach antigenic sites located at the base of the spikes or on the surface of the lipid layer because the inter spike distance is approximately 60 Å In the present experiments neither whole anti F serum nor concentrated IgG and IgM fractions showed any HI effect (Table 1) The HA material in the IB light fraction (Fig 1) however was inhibited by anti F serum Considering the density of intact virus particles which is 1 22 g/cm³, this light HA material must be composed of viral membranes or transformed host membranes which allow F antibodies to block the HA This is possibly caused by aggregation of the material thus indicating exposed F determinants

The absorption experiments with virus preparations of different degree of purity showed that with more extensive purification the capacity to absorb anti F antibodies finally became non detectable (Table 2) These findings are supported by the experi

ments with Sepharose 6B (Figs 3 and 4) showing that purified virus (O/DC/IB) in contrast to crude virus (O/DC) did not re duce the haemolytic activity of the serum eluted fractions Our findings are in contrast to those of *Drzeniek et al* (2) *Rott et al* (9) and *Drzeniek* (1), who purified the virus by differential centrifugation In our hands this did not separate the virus from HA active and F positive contaminants. Purification by velocity centrifugation combined with isopyc nic banding or only by the latter method made the removal of these contaminants pos sible It is seen from Figs 1 and 2 that the ap parently homogeneous HA material isolated after velocity centrifugation is split into HA material of d fferent densities by isopycnic banding The HA active and F positive ma terial in fraction 12 (Fig 2) can therefore not represent intact virus particles which have a buoyant density of 1 22 g/cm³ Whe ther this l ght HA material is residual ma terial from degraded virions or is composed of tranformed host membranes is uncer tain The existence of F determinants in the influenza particles is therefore still uncertain The same purified virus preparation absorbed antibodies to the host antigen and the homologous A_0/PR8 antigens

It is possible to calculate roughly the num ber of antibody molecules and virus particles involved in these reactions According to *Fazekas de St Groth & Webster* (3) a maxi mal antigenic stimulation results in approxi mately 10^{15} specific antibody molecules/ml serum In this experiment (Table 3) there should be approximately $2\ 5 \quad 10^{5}\ (16\,384)^{1}$ $\sim 15 \quad 10^{0}$ anti F antibody molecules in the reaction mixture Furthermore according to *Fenner & White* (4) there will be approxi mately $2\ 5 \quad 10^{10}$ virus particles in the same mixture

The average avidity of the anti F anti bodies is arbitrarily represented by an equili brium constant (of formation) of the anti gen antibody complexes $K = 10^{12}$ (in cgs units a moderate estimate) The lowest average number of antibodies attached to each virion is one and this will as a conse quence of the sensitivity of these HL reac tions result in a drop in the HL-titre after the absorption of 1 log step which was not observed here By the same calculations one antibody/virion equals approximately 40 F sites This is also highly improbable because one must be allowed to assume that the F determinants if presents must be located to either one or both of the two different virus spikes (*i e* the haemagglutinin and neura minidase spikes) A low estimate of the num ber of neuraminidase spikes is about 500, as compiled from literature data Therefore considering the uncertainties in these calcula tions it is improbable that antibody acces sible F determinants exist at the viral sur face

The same calculations applied to the ab sorption experiments with anti B/Lee and anti A_0/PR8 sera indicate that between 1 000 and 2 000 antibody molecules must be bound to each particle in order to give the observed titre reduction This is in agree ment with the calculations made by *Fazekas de St Groth & Webster* (3), who found that each influenza virus particle has approxi mately 2 000 well separated antigenic sites

We therefore conclude that Forssman de terminants are not exposed at the surface of egg grown influenza virus Studies are in progress to investigate whether these deter minants are present in the virion

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CANINE MYCOPLASMAS I: CULTIVATION FROM CONJUNCTIVAE, RESPIRATORY- AND GENITAL TRACTS

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The occurrence of mycoplasmas in the conjunctivae and in the respiratory and genital tracts of dogs has been investigated by cultivation experiments Whereas mycoplasmas from conjunctiva could be cultivated in a few cases only, they occurred in a very high frequency in the upper respiratory tract without relation to clinical disease Mycoplasmas from lungs with pneumonic lesions were cultivated but not mycoplasmas from normal lungs From the genital tract, they were isolated from half of the female dogs, and a little more frequently from the males Using indirect immunofluorescence identification of mycoplasma colonies, a mixed flora was found in the pharynx of approx 85 per cent of the examined dogs In half of the female dogs a mixed flora was present in the vag na whereas this was apparently not the case in the prepuce of male dogs

The first systematic investigation of mycoplasmas in dogs was published in 1951 (6) The isolated strains were classified into three serologically distinct groups designated α, β and γ One strain (C 21) could not be classified within any of these groups In 1956 the groups were species named *M spumans, M canis* and *M maculosum* (7) Strain C 21 was recently classified together with a number of new isolates as a fourth canine species *M edwardii* (14)

During an investigation of pneumonia in dogs in a laboratory colony *Armstrong et al* (1) isolated mycoplasma strains belonging to the said four species A few additional strains were isolated which could not be identified These strains were placed in two sero groups C and D *Barile et al* (2) isolated mycoplasmas from larynx and the genital tract of dogs The mycoplasma colonies were identified by direct immunofluorescence on agar plates, this method disclosed a high frequency of a mixed mycoplasma flora consisting of two or more species In addition, one strain (HRC 689) was isolated which could not be identified Eleven mycoplasma strains isolated as part of the present study were previously classified as a new species, *Mycoplasma cynos* (12)

The purpose of this investigation was to examine the occurrence of mycoplasmas in dogs by attempted cultivation of specimens from the conjunctivae and the respiratory and genital tracts, with particular reference to the prevalence of a mixed flora

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MATERIALS AND METHODS

1 *Cultivation Experiments*

a *Dogs Group 1 (dogs 1 32)* It was attempted to cultivate mycoplasmas from 32 dogs with no signs of disease in the conjunctivae or the respiratory and genital tracts Seven dogs originated from isolated milieus (private), whereas the rest were from crowded milieus (a kennel and an animal hospital)

Group 2 (dogs 33 55) This group consisted of dogs from crowded milieus The dogs were suffering from respiratory diseases of infectious type at the time of the cultivation experiments

Group 3 (dogs 56 70) In this group all 15 dogs which were healthy originated from a kennel The dogs were killed, post mortem examination performed just prior to cultivation experiments revealed no macroscopic lesions in the organs

Group 4 (dogs 71 78) The group consisted of 8 dogs from the same kennel as those in group 3 Post mortem examination of the lungs revealed pneumonic areas of varying extent The pneumonias were histologically of the interstitial type with infiltrations dominated by lymphocytic cells. In a single dog (no 71) also slight infiltration with eosinophilic granulocytes was seen around the bronchioli In two dogs (nos 77 and 78) distemper was diagnosed on the basis of demonstration of inclusion bodies in epithelial cells from conjunctiva, trachea and vesica

b *Culture media* B Heart infusion broth (Difco) 2 5 per cent (w/v) 90 ml horse serum 20 ml fresh yeast extract 25 per cent (w/v), 10 ml DNA (Sigma) 0 2 per cent (w/v) 1 2 ml 2,3 5 triphenyl tetrazolium chloride, 1 per cent (w/v) 1 0 ml thallium acetate, 10 per cent (w/v), 1 0 ml Na penicillin 50 000 i u Solid medium was prepared by substitution of heart infusion broth with heart infusion agar (4 per cent, w/v)

N Brain heart infusion broth (Difco) 3 7 per cent (w/v) 100 ml horse serum, 20 ml yeast extract (Difco) 0 5 g fresh yeast extract 25 per cent (w/v) 10 ml DNA (Sigma) 0 2 per cent (w/v), 1 2 ml, glucose, 1 0 g thallium acetate 10 per cent (w/v), 1 0 ml Na penicillin 50 000 i u Solid medium was prepared by adding 1 7 g of ionagar No 2 (Oxoid)

For both media semisolid substrates were prepared by adding 0 1 g ionagar No 2 The pH of the media was adjusted to 7 5

As regards dogs in the groups 1 and 2, cultivation of specimens was done on B-medium In the case of group 3, cultivation of specimens from 7 dogs (nos 56 62) was made on B-medium and of specimens from 8 dogs (nos 63 70) on both B and N medium In group 4 cultivation of specimens from 4 dogs (nos 71 74) was attempted on both B and N medium, whereas B medium only was used for specimens from the remaining 4 dogs (nos 75 78)

c *Cultivation technique* Specimens from dogs in groups 1 and 2 were collected by sterile cotton swabs The swab was rubbed against the mucosal [illegible] and finally [illegible]

membranes of conjunctivae and the other tissues examined The surface of the membrane specimens was smeared onto the solid medium and then deposited in semisolid medium In the case of lungs without pneumonia cultivation was attempted on specimens from the ventral part of the right or left apical lobe In lungs with pneumonia a piece of the pneumonic area was excised smeared onto solid medium and then placed in semisolid medium

Incubation was performed aerobically at 37° C in a humid atmosphere The plates were incubated 4 days before examination for growth If no colonies were found the plates would be incubated for a further 4 days From the semisolid media subcultivation on solid media of identical composition was performed every second day for 8 days

Cloning was initiated when growth appeared on the plates Single colonies were cloned three times. The cloned culture was stored at −70° C for subsequent classification studies

TABLE 1 *Recovery Rate of Mycoplasmas from Dogs (Groups 1 and 2)*

	Group 1 (Nos 1 32) (no respiratory or genital disease)		Group 2 (Nos 33 55) (respiratory disease)	
	No of positive cultures	No examined	No of positive cultures	No examined
Conjunctiva	1	8		
Pharynx	31	32	23	23
Vagina	6	13	2	3
Prepuce	6	12	5	5

TABLE 2 *Recovery Rate of Mycoplasmas from Dogs (Groups 3 and 4)*

	Group 3 (Nos 56 70) Normal dogs		Group 4 (Nos 71-78) Dogs with pneumonia	
	No of positive cultures	No examined	No of positive cultures	No examined
Conjunctiva	2	9	1	2
Larynx	6	7	2	2
Trachea	2	2	3	3
Lung	0	15	7	8
Cervix	3	7	1	1
Prostate	1	2	1	1

II Examination for Mixed Mycoplasma Flora

The examination was based on the identification of mycoplasma colonies using indirect immunofluorescence (11) Antisera against the type strains of the following species were used *M spumans, M canis, M maculosum* and *M edwardii* The growth area on the agar plates was cut into four identical blocks, each block being treated with one antiserum In order to avoid possible cross reactions, the sera were used in their optimal dilutions which means the highest dilution giving strong fluorescence in a homologous titration Only cultures on B plates were examined Plates, inoculated on the second day, from semisolid medium (*secondary plates*) *were used from 52 cultivations* Of these 52 cultivations, 15 plates inoculated directly with the swab (primary plates) were examined as well With a view to controlling the serum and fluorescence intensity, each antiserum was tested against colonies of the homologous type strain Plates were regarded positive if colonies with the same fluorescence intensity as control colonies were found

RESULTS

I Cultivation experiments The results of cultivation of specimens from dogs in groups 1 and 2 are given in Table 1 Cultivation from the conjunctivae was positive (*M canis*) in only one out of eight attempts Mycoplasmas from the pharynx of practically all dogs could be cultivated, whether the dogs were normal or suffering from respiratory disease Cultivations from the vagina were positive in about half of the cases, this was

prepuce

Table 2 gives the results for dogs in groups 3 and 4 Mycoplasmas could be cultivated from the conjunctivae of three (*M spumans, M cynos,* and *M bovigenitalium*) out of eleven dogs, and from the larynx and trachea in nearly all dogs examined

No growth was obtained from the lungs of normal dogs, whereas mycoplasmas could be cultivated from pneumonic tissues in seven out of eight cases of pneumonia (2 *M spumans*, 1 *M edwardii*, 1 *M cynos*, 1 *M bovigenitalium*, and 2 *M feliminutum* strains) Included in this group were the two dogs (nos 77 and 78) with distemper Mycoplasmas from the mucosal membrane of the cervix were cultivated in four of eight dogs, and from the prostate in two out of three dogs

In 12 cultivation attempts where B- and N-medium were used simultaneously, growth was confined to the N medium in one case only This was the cultivation of specimens from the conjunctiva and of pneumonic tissue from dog no 71, yielding growth of an organism identified as *M bovigenitalium*

II Mixed mycoplasma flora The results of studies of the composition of the mycoplasma flora appear from Table 3 A mixture of 2, 3, or 4 species was found in the pharynx of 30 out of 35 (approx 85 per cent) cultures examined In the vagina, a mixed flora was demonstrated in almost half of the dogs whereas no mixed flora was found in the prepuce Colonies identified as *M maculosum* were as a rule scanty and lacking the characteristic fried-egg morphology In several cultures, especially of specimens from the

TABLE 3 *Composition of Mycoplasma Flora in Dogs*

	Source of cultivation	Number of cultivations	Number of cultures containing					Number of cultures containing			
			0 species	1 species	2 species	3 species	4 species	*M spumans*	*M canis*	*M maculosum*	*M edward*
nary es	Pharynx	11	1	2	4	4	0	3(27%)	5(45%)	5(45%)	9(8[illegible]
	Vagina	2	2	0	0	0	0	0	0	0	0
	Prepuce	2	2	0	0	0	0	0	0	0	0
ondary es	Pharynx	35	0	5	8	15	7	15(43%)	29(83%)	22(63%)	28(80%
	Vagina	7	1	3	3	0	0	1	5	3	0
	Prepuce	10	4	6	0	0	0	1	4	0	1

vagina and prepuce yielding growth of mycoplasma colonies, none of these could be identified as either *M spumans, M canis, M maculosum* or *M edwardii* Neither could cloned strains from these cultures be classified as any of the 4 known species

Of the 4 species looked for, *M canis* and *M edwardii* predominated in the pharynx, although *M spumans* and *M maculosum* were also quite frequent here In the vagina, all species except *M edwardii* were demonstrated, and in the prepuce all except *M maculosum* The incidence of *M spumans, M canis* and *M maculosum* as demonstrated on secondary plates was significantly higher than that of the same species on primary plates On the other hand *M edwardii* was found with the same frequency on both primary and secondary plates

DISCUSSION AND CONCLUSIONS

Cultivation The media designated B and N seem to be equally suitable for the cultivation of mycoplasmas from dogs

The isolation of mycoplasmas from the conjunctivae of dogs has apparently not been described earlier The almost consistent demonstration of mycoplasmas in the upper respiratory tract is in agreement with the findings by *Edward & Fritzgerald* (6) *Brennan & Simkins* (4), and *Binn et al* (3) The overall incidence of mycoplasmas in the pharynx of dogs suffering from respiratory diseases did not differ from that of normal dogs neither was there any difference in this respect between dogs originating from crowded and isolated milieus

The isolation of mycoplasmas from pneumonic tissues in several cases is in accordance with the observations by *Armstrong et al* (1), who apparently did not, include healthy controls in their study

An earlier study by the present author (10) suggested that *M cynos* might be a cause of pneumonia in dogs In this study, the high rate of recovery of mycoplasmas (*M spumans, M edwardii M cynos, M bovigenitalium,* and *M felim nutum* strains) from the lung tissues of dogs with pneumonia, but not from normal dogs provides further evidence in support of the assumption that mycoplasmas may play a role in the development of pneumonia in dogs either as primary pathogens or as secondary invaders On the other hand, the association of several different *Mycoplasma* species with pneumonia would seem to call for some caution in the evaluation of the possible significance of these findings As to the *M bovigenitalium* strain isolated from dog no 71, this might very well have been the primary pathogen Some strains, at least, of *M bovigenitalium* are pathogenic for cows and bulls, and the lesions produced are characterized by the infiltration

of eosinophilic granulocytes (8) just like those demonstrated in the pneumonic focus in dog no 71

The isolation of two strains of *M feliminutum* deserves particular attention inasmuch as only one isolation of this species, from the pharynx of a cat, has been reported previously (9)

The recovery rate of mycoplasmas obtained in this study, in female dogs is at about the same level as that found by *Edward* & *Fitzgerald* (6) The incidence in the prepuce is somewhat smaller than that reported by *Chu* & *Beveridge* (5) However, their results were based on dogs suffering from chronic balanoposthitis

Mixed mycoplasma flora Because of the specificity of the indirect immunofluorescence method, it is very suitable for the identification of mycoplasmas in mixed culture

In about 85 per cent of the dogs examined, more than one species could be demonstrated in cultures from the pharynx. In the vagina a mixed flora was found in barely half of the dogs, whereas no mixed flora was found in prepuce The results presented here are on the whole in agreement with those obtained in a similar investigation utilizing the direct immunofluorescence technique (2)

The higher isolation rate of *M spumans, M canis,* and *M maculosum* on secondary plates as compared to primary plates shows that the former are superior to primary plate cultures for the study of materials containing a mixture of different mycoplasmas

The biochemical and serological characterization of the isolated strains will be described in a subsequent paper (13)

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EVALUATION OF TWO DIFFERENT ANTIGEN PREPARATIONS IN THE FLUORESCENT ANTIBODY TEST FOR ANTINUCLEAR ANTIBODIES (ANA)

A comparison between two laboratories

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Two different antigen preparations of human origin (sectioned thyroid cells and blood smears) were compared in the indirect immunofluorescence (IF) test for antinuclear antibodies (ANA) Two sets of sera were investigated with identical reagents at two laboratories equipped with similar optical systems The reproducibility was 94 per cent if thyroid sections were used at both places but dropped to 72 per cent if one laboratory substituted thyroid sections for blood smears All sera showing speckled staining with thyroid sections were negative with blood smears Tests carried out on blood smears resulted consistently in higher ANA titres as compared with results obtained with thyroid sections Low ANA titres were found in 5 per cent of healthy blood donors if blood smears were used but only in 1 per cent with sectioned thyroid cells Tissue sections are recommended for routine screening of ANA

The LE cell phenomenon in systemic lupus erythematosus (SLE) was discovered in 1948 by *Hargraves et al* (10), but it was not until 1954 that *Miescher & Fauconnet* (16) could show that it is caused by antinuclear antibodies (ANA) It was later found that the responsible antibodies can be directed against different constituents of the nucleus There exist at least four different kinds of ANA which are directed against DNA (12) RNA (3), histone (15) or glycoprotein (13) respectively These different types of ANA are usually found individually or in combination in sera from patients with diseases of auto immune origin Well known examples are SLE, rheumatoid arthritis dermatomyositis, systemic sclerosis, Sjögrens syndrome, and chronic active hepatitis

While the LE-cell phenomenon is the classical test for ANA in SLE a number of serologic techniques have been developed for the detection of ANA in general Such methods are antiglobulin consumption (17), precipitation (5), passive agglutination (18), complement fixation (5), and immunofluorescence (IF) (1, 8, 11, 12) Introduced in 1941 by *Coons et al* (6), IF has undergone several modifications such as the introduction of the indirect technique in 1954 (24) Most laboratories performing routine examinations for ANA use the indirect IF test because of its simplicity and sensitivity

Indirect IF testing for ANA requires a two-step procedure before microscopy can take

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place Patient sera are incubated with nucleated cell material such as smears or tissue sections followed by incubation with fluorochrome tagged anti human antibodies (conjugate) Nuclei from a variety of sources have been used with good results Such antigen preparations include blood or cell smears and tissue sections from different organs of vertebrates (2)

In order to obtain comparable results in different laboratories and even from time to time in one individual laboratory, reagents and instruments have to be carefully assessed Much work has been carried out in this respect especially with regard to the microscope (14) and the conjugate (4) This work was designed to study the reproducibility of (positive and negative) results obtained in two laboratories and to show the influence on the results of the antigen preparation

MATERIALS AND METHODS

Scope of Investigation

Two laboratories with experience in ANA tests with indirect IF technique took part in the investigation They will be referred to as Laboratory A and Laboratory B in the following text

A total of 67 sera were randomly drawn out of the files at the two laboratories Laboratory A contributed with 40 sera while Laboratory B selected 27 sera. These two sets of sera were coded interchanged between the two laboratories and investigated at both places In addition 100 serum specimens from blood donors were collected by laboratory B and tested independently by the two laboratories

Antigen Preparation

Human thyroid sections (HTS) and human blood smears (HBS) were used The HTS were obtained from thyroid glands of patients who were operated on for diffuse toxic goitre Pieces of the glands of about 5 mm × 5 mm were snap frozen in a mixture of dry ice and acetone or with the use of liquid CO The pieces were stored in the freezer (−63° C) until they were cut in a Harris M 40 cryostat Harris Refrigeration Co Massachusetts USA) that was operated at −18° C Frozen sections 6 8 microns thick were placed on well cleaned glass slides thawed immediately dried in a stream of air at room temperature for one hour and fixed in acetone for 10 minutes The slides were then dried at room temperature and used within 4 days If not used the same day the slides were kept in the refrigerator

The HBS were prepared from drops of finger tip blood that were smeared on well cleaned glass slides with the use of glass cover slips The slides were dried at room temperature and before use a circular area of about 15 20 mm was etched in the tail of the smear The smears were then stored in the refrigerator and used within 4 days

Conjugate

A commercially produced heterospecific sheep anti human conjugate, batch no SH 75103 (NBL, Stockholm, Sweden), was used in all investigations The conjugate parameters according to the manufactures were 58 2 µg/ml of fluoresceinisothiocya[illegible]nate, 7 0 mg/ml of total protein, 3 0 mg/ml of specific anti IgG antibody and a weight fluorochrome to protein ratio of 6 2 × 10^{-3}

Performance of the ANA Test

Serum specimens were initially diluted 1 10 in 0 01 M phosphate buffered saline, pH 7 8 (PBS) One drop (approximately 0 003 ml) from each serum dilution was applied to slides, incubated a moist chamber at room temperature for 20 [illegible] minutes rinsed with PBS and washed for 10 minutes. One drop of diluted conjugate was then applied to each slide and the procedure was repeated The preparations were finally mounted under cover glass slips with PBS buffered glycerol of pH = 7 8 (1 part PBS and 9 parts glycerol) as mounting fluid

Appropriate controls were included each test day They included known positive and negative serum specimens as well as incubation with the conjugate alone

Microscopy

Both laboratories used a standard fluorescence microscope model RA II (Carl Zeiss, Oberkochen, West Germany) equipped with a Osram HBO 200 lamp as light source For reading the slides Laboratory A used a dry dark field condenser with a 3 mm BG3 primary filter in combination with secondary filter no 44 Laboratory B used a dark field oil immersion condenser with a 3 mm UG5 primary filter in combination with secondary filter no 41 The thyroid sections and the blood smears were examined at a magnification of 125 × or 250 ×

RESULTS

Evaluation of Sera Selected by Laboratory A

Table 1 summarizes the results of a comparison of findings obtained in the two labo-

TABLE 1 *Comparison of the Results Obtained by the Two Laboratories A and B Sera Selected by Laboratory A*

Laboratory A Results of ANA tests with HTS (Thyroid sections)		Laboratory B Results of ANA tests with			
		HTS (Thyroid sections)		HBS (Leucocytes in blood smears)	
		Positive	Negative	Positive	Negative
Positive	20	19	1	14	6
Negative	20	0	20	1	19
Total	40	19	21	15	25

ratories by tests of sera selected by Laboratory A Sera were tested with HTS by Laboratory A and then re-investigated by Laboratory B with HBS and HTS

It can be seen that out of 20 ANA positive sera in Laboratory A six were negative in Laboratory B if HBS were used as antigen while only one was negative if HTS were used One of those that were negative in Laboratory A was positive in Laboratory B with HBS but not with HTS

Evaluation of Sera Selected by Laboratory B

Table 2 summarizes the results of a comparison of findings obtained in the two laboratories by tests of sera selected by Laboratory B Sera were tested with HBS and HTS at Laboratory B The procedure was repeated at Laboratory A but only with HTS

It can be seen that out of the 20 sera that were positive in Laboratory B with HBS one was considered negative by the two laboratories with the use of HTS while the other 19 were considered positive None of those negative with HBS were positive with HTS

Comparison of ANA Titres Obtained in the Two Laboratories with HBS and HTS

A comparison of ANA titres obtained in the two laboratories with HTS is shown in Fig 1

It can be seen that only three serum specimens with fourfold deviation of the ANA titres were obtained in the two laboratories while the rest of the titres were within the error of titration Fig 1 also shows that the serum specimen positive in Laboratory A but negative in Laboratory B (Table 1) had a titre of only 1 10

Fig 2 shows a comparison between the ANA titre obtained in Laboratory A with HTS and that obtained in Laboratory B with HBS It can be seen that out of the 33 serum specimens giving positive ANA tests with HTS and HBS 11 gave a four to eightfold higher titre with HBS The two serum speci

TABLE 2 *Comparison of the Results Obtained by the Two Laboratories A and B Sera Selected by Laboratory B*

Laboratory B Results of ANA tests with HBS (Leucocytes in blood smears)		Results of ANA tests with HTS (Thyroid sections)			
		Laboratory A		Laboratory B	
		Positive	Negative	Positive	Negative
Positive	20	19	1	19	1
Negative	7	0	7	0	7
Total	27	19	8	19	8

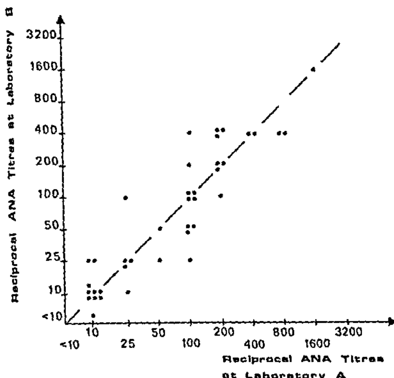


Fig 1 Comparison of ANA titres observed in the two laboratories A and B using human thyroid sections (HTS)

mens positive with HBS but negative with HTS, had titres of 1 10 and 1 25 respectively In five out of six sera (83 per cent), positive with HTS but negative with HBS, a speckled staining pattern was noted by both laboratories Titres ranged from 1 10 up to 1 400 Only one of these sera showed a homogenous staining (1 10) All other sera, positive with HTS and/or HBS, gave a homogenous staining pattern at both laboratories

Comparison of ANA Tests with Blood Donors Sera

The results of ANA tests performed by the two laboratories on serum specimens from 100 blood donors are summarized in Table 3

It will be seen that one blood donor gave a positive ANA test with HTS in both laboratories The titre of the reaction was 1 10 This blood donor s serum also reacted with HBS at a titre of 1 25 In addition, another five blood donors sera reacted with HBS three of these with a titre of 1 10 and two with a titre of 1 25

DISCUSSION

The use of indirect IF technique for the detection of ANA in the serum of patients with SLE, rheumatoid arthritis, systemic sclerosis and other systemic diseases is now routine in many laboratories A wide variety of microscopic equipment and nuclear antigen preparations as well as conjugates prepared in a variety of animals, are utilized These variations in methodology are undoubtedly responsible for some of the differences in reported results

This investigation was performed by two laboratories using similar optical systems Differences in filter combinations and condensers did not affect the result significantly (Fig 1), which is in accordance with earlier findings (9) Only four of the 67 sera examined showed inacceptable variations, which

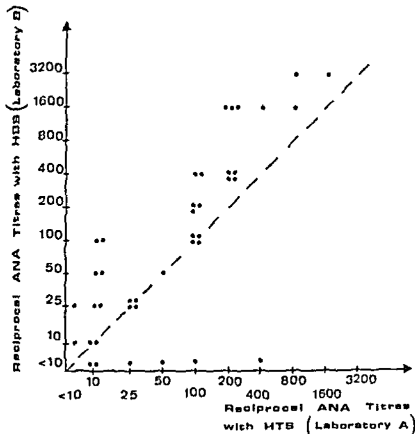


Fig 2 Comparison of ANA titres obtained in Laboratory A using human thyroid sections (HTS) and in Laboratory B using human blood smears (HBS)

lends the present study a reproducibility of 94 per cent if both laboratories used human thyroid sections (HTS) as antigen preparation and the same conjugate The reproducibility dropped, however, to 72 per cent (Fig 2) if two different antigen preparations HTS versus human blood smears (HBS) were used This discrepancy was obvious even if the two antigen preparations were used within one laboratory (Table 1) The differences between these results clearly indicate the need of standardization of nuclear antigens used for the detection of ANA in serum

Some observations concerning the staining pattern in HTS and HBS merit special comments

TABLE 3 *Comparison between the Two Laboratories A and B of ANA Tests of 100 Blood Donors Sera*

Laboratory A Results of ANA tests with HTS (Thyroid sections)		Laboratory B Results of ANA tests with HTS (Thyroid sections)		Laboratory B Results of ANA tests with HBS (Leucocytes in blood smears)	
		Positive	Negative	Positive	Negative
Positive	1	1	0	1	0
Negative	99	0	99	5	94
Total	100	1	99	6	94

There was a 100 per cent agreement between the ANA positive sera that gave a homogenous staining pattern with HTS and HBS in dilutions of 1 25 It was found, however, that out of six serum specimens giving positive ANA with HTS but negative with HBS, the discrepancy was due to a speckled staining pattern in five of the specimens After the results with HTS were known, repeated tests with these sera were performed with smears of HBS from various donors It was now found that two of the sera actually gave a speckled staining pattern with some, but not all, leucocytes The staining was much more discrete than that obtained with HTS and was easily missed because of its irregular occurrence At present, we do not know whether this is due to an extractable antigen, but the use of unfixed or acetone fixed HBS did not influence the results

The findings indicate that blood smears are less suitable for the detection of nuclear antigens showing speckled staining We have also found them unsuitable for the nucleolar staining pattern (unpublished observations) No specific patterns occur with formalinized chicken erythrocytes which have been proposed by *ten Veen* & *Feltkamp* (23) as an easy and reliable antigen for ANA detection In contrast to the here reported results obtained with HBS these authors noted no difference (titre changes) between titres in liver sections and chicken erythrocytes Instead, specific patterns were simply replaced by a uniform homogenous annular fluorescence (23)

Faber et al (7) have reported on the occurrence of a granulocyte specific ANA in patients with Felty s syndrome This antibody reacted with human granulocytes but not with HTS This has been confirmed by *Szec* (22) who also found granulocyte specific ANA to occur especially in patients with rheumatoid arthritis She differentiated between two separate granulocyte specific ANA one of which showed a significant association with granulocytopenia typical of Felty s syndrome Therefore the use of HBS should not be completely banned but be utilized on special indications

In this study, consistently higher titres were obtained with HBS as compared with HTS This fact could be explained by qualitative differences between available nuclear antigens, but is more likely caused by the obvious quantitative differences The number of nucleated cells in a tissue section is higher by several hundreds than that in a blood smear, and there are no easily revealed qualitative differences between nuclear antigens in leucocytes and in other cells (19) The control group of 100 blood donors' sera showed a 100 per cent reproducibility if HTS was the antigen preparation Only one low titre ANA serum was found at both laboratories On the other hand, five additional positive reactions were seen at laboratory B when tests on HBS were attempted

The higher yield of ANA positive sera with HBS among blood donors (5 per cent) indicates a relatively high proportion of ANA in sera from apparently healthy persons This contrasts with the findings obtained if HTS was used as antigen (1 per cent) According to several reports (1, 20 21, 25), tests on tissue sections have shown that a certain proportion of the normal population contain low titres of ANA in their sera It is obvious that certain levels of significance must be accepted in order to differentiate between normal and pathological conditions *Ritchie* (20) attaches no diagnostic significance to titres below 16, while others (25) put the level lower and in addition differentiate between sex and age It is more than probable that HBS should have revealed considerably higher frequences, making the problem of significance elusive

This study indicates a good reproducibility of ANA test with the use of HTS However, the antigen preparation must not necessarily consist of human thyroid sections Laboratories which do not have an easy access to thyroid glands have in many cases used other tissue sections such as rat or mouse liver sections with good results (2 3, 9 20) In order to simultaneously reveal a variety of autoantibodies laboratory A has included sections

of rat kidney and rat stomach together with a thyroid section in its routine testing programme It has been the experience that ANA can also be seen in other sections if it is present in the thyroid section Although the presence of specific ANA in blood smears indicates the existence of at least one antigen exclusive for leucocyte nuclei the vast majority of ANA will react with any nuclei regardless of their origin On these grounds, tissue sections are recommended as the antigen of choice for routine testing of sera suspected to contain ANA

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LOCALIZATION OF HORSE RADISH PEROXIDASE IN MOUSE AND GUINEA PIG PERITONEAL MACROPHAGES AFTER UPTAKE *IN VIVO* AND *IN VITRO*
AN ELECTRON MICROSCOPE STUDY

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Normal mouse and guinea pig macrophages which had ingested horse radish peroxidase (HRP) were studied in the electron microscope The majority of the HRP was situated in the phagolysosomes 10, 30 60 minutes and 24 hours after injection *in vivo*, or *in vitro* at the shorter time intervals The localization of ^{125}I HRP in macrophages was also studied at the electron microscope level Radioactivity was usually associated with the histochemical staining for peroxidase which was chiefly situated in the phagolysosomes Occasional grains were found on the surface of the cell The significance of these findings is discussed in the context of the role of macrophages in the induction of the immune response

Horse radish peroxidase (HRP) has been employed to trace the fate of antigen in macrophages by electron microscopy (*Catanzaro et al* 1969, *Cotran & Litt* 1970) *Catanzaro et al* found HRP to be localized in the endoplasmic reticulum and the perinuclear envelope of guinea pig peritoneal macrophages In these sites the antigen would presumably be protected from breakdown in contrast to that which is found in the phagolysosomes. However, *Cotran & Litt* found no such localization of exogenous peroxidase in peritoneal macrophages from guinea pigs Rather their experiments suggested that the peroxidase which was found in the endoplasmic reticulum and nuclear membrane was endogenous and that exogenous peroxidase was localized in phagolysosomes as is the case with other antigens (*Nossal et al* 1968, *Rhodes e al* 1969, *Unanue et al* 1969)

This report deals with the fate of HRP in peritoneal macrophages from mice and guinea pigs, *in vivo* and *in vitro* The results largely confirm those of *Cotran & Litt* (1970), *Anteunis et al* (1972) and *Steinman & Cohn* (1972) In addition the uptake of ^{125}I peroxidase in mouse peritoneal macrophages was studied *in vitro*, according to the method of *Unanue et al* (1969)

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MATERIAL AND METHODS

Animals

Inbred C3H mice of both sexes 4-5 months old bred at the Statens Seruminstitut and random bred

guinea pigs of both sexes 400 160 g bred at the Statens Seruminstitut, were used

Antigen

Horse radish peroxidase (HRP) type II and type VI were purchased from Sigma Chemical Co, St Louis USA

Reagent

3,3 diamino-benzidine tetrahydrochloride was obtained from Fluka Ltd, Buchs, Switzerland

Iodination

HRP type VI was labelled with carrier free ^{125}I according to the method of *Hunter & Greenwood* (1962) at a specific activity of 0.5 μCi/μg The carrier free ^{125}I was obtained from the Radiochemical Centre, Amersham England

Harvesting and Treatment of Peritoneal Exudate Cells

Mice were first injected intraperitoneally (ip) with 2 ml of 10 per cent proteose peptone in saline 3 days prior to the experiments Guinea pigs were injected with 8 10 ml 10 per cent proteose peptone ip

In vivo Experiments

Mice treated as above divided into groups of 20 were injected with 1 mg or 10 mg HRP/ml ip Controls were injected either with saline or not at all The mice were killed 10 30 or 60 minutes after injection and control mice 60 minutes after injection

The cells were harvested by washing out the peritoneal cavity with 3 ml saline containing heparin 15 IU and 5 per cent normal rabbit serum The cells were centrifuged at 700 *g* for 3 minutes and then washed twice in Gey's solution and once in saline 5 ml 1.5 per cent glutaraldehyde in cacydolate buffer ph 7.2 was added and the cell suspension was left for 10 minutes The cells were then washed twice with 0.2 M sucrose in cacydolate buffer pH 7.2 suspended in 5 ml of this solution and then stored overnight at 4° C The cells were centrifuged and 10 ml 0.05 M TRIS hydrochloric acid pH 7.6 containing 5 mg 3.3 diamino-benzidine tetrahydrochloride and 0.01 per cent hydrogen peroxide were added The suspension was left for 10 minutes and the cells were centrifuged at 700 *g* for 5 minutes and washed with 3 × 5 ml sucrose-cacydolate buffer Subsequently the cells were prepared for electron microscopy as described previously (*Rhodes et al* 1969) except that a low viscosity epon (*Spurr* 1969) was substituted for Vestopal W

Six guinea pigs per group were also treated as above the cells being harvested from the peritoneum with about 20 ml heparin 15 IU containing 5 per cent normal rabbit serum Otherwise the procedure was as stated above Guinea pig cells were also harvested 24 hours after injection

In vitro Experiments

Peritoneal exudate cells from mice or guinea pigs were harvested as above 3 days after a prior injection of proteose peptone Cells were harvested in heparin serum containing 30 IU penicillin per ml The cells were washed in Gey's solution containing 10 per cent normal rabbit serum 30 IU penicillin per ml and 100 μg streptomycin per ml (medium)

About 2 × 10^{8} cells were suspended in 50 ml medium and either a) 1 mg HRP/10^{7} cells, or b) 10 mg HRP/10^{7} cells were added The cell suspensions a) and b) were incubated for 10 30 and 60 minutes After incubation all cells were washed twice with medium and once with saline 5 ml 1.5 per cent glutaraldehyde was added to the cells and these were then further treated as described under *in vivo* experiments

Radioactive Experiments

Peritoneal exudate cells were obtained as described above from mice injected 3 days previously with proteose-peptone

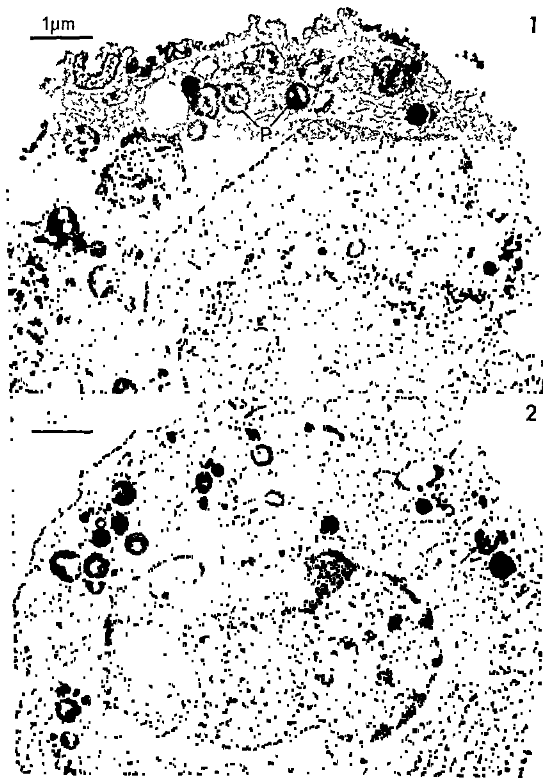
10^{7} cells were incubated with 1 mg ^{125}I peroxidase in a plastic screwcap jar in 6 ml Parker's medium containing 30 IU penicillin per ml, 100 μg streptomycin per ml and 10 per cent normal mouse serum The mixture was shaken slowly at 37° C in a CO_2/O_2 atmosphere for one hour The cells were washed three times with Parker's medium and the amount of radioactivity associated with the cells was determined The cells were then resuspended in 10 ml medium devoid of antigen and incubated at 37° C for 3 hours under the same con

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All figures show electron micrographs of sections of cells obtained from the peritoneum of proteose-peptone stimulated mice or guinea pigs As stated in MATERIAL AND METHODS glutaraldehyde pre-fixation and osmium post fixation were used throughout Unless otherwise stated no counter staining of sections was employed

Fig 1 Macrophages from a pool of cells obtained from mice injected with 10 mg HRP ip The cells were harvested 10 minutes after injection HRP reaction products are numerous in the phagolysosomes (P) between the two cells and on the surface of the cells × 16 000

Fig 2 A macrophage from a pool of cells obtained from mice injected with 1 mg HRP ip The cells were harvested 30 minutes after injection HRP reaction products can clearly be seen in the phagolysosomes (P) × 16 000


1µm
1
O
P
2

100nm
P
CM
3
100nm
P
CM
4

ditions as above They were then centrifuged washed three times with medium and the radio activity associated with the cells was again deter mined An aliquot of the cells was centrifuged onto slides using a cytocentrifuge (*Doré & Balfour* 1965) and Ilford L4 emulsion was used to coat the slides Exposure time was 14 days The remaining cells were fixed in glutaraldehyde then prepared for electron microscopy and subsequent autoradio graphy using Ilford L4 emulsion and a technique exactly as described previously (*Rhodes et al* 1969) The sections were exposed for 14 days

Two controls were included in this experiment a) 10^7 cells exposed to 1 mg non radioactive HRP type VI and b) 10^7 cells incubated in medium Both sets of cells were treated in the same manner as the radioactive cells

RESULTS

Ultrastructural Localization of HRP

The results from experiments with both mice and guinea pigs were so similar that they will be considered together

In vivo

10 minutes after i p injection HRP was found attached to the cell membrane, in the phagolysosomes and trapped between the cells, particularly when 10 mg was injected (Fig 1) By 30 and 60 minutes large quantities of HRP had been taken up into the phagolysosomes, this was the most characteristic pattern observed in all the experiments (Fig 2)

After ½-1 hour s incubation not many cells had HRP on the cell surface, but there was a small percentage from both mice and guinea pigs which had HRP adhering to the cell membrane, particularly from those animals which had received 10 mg peroxidase (Fig 3)

Control cells from mice and guinea pigs incubated without HRP showed little reaction for peroxidase Some cells contained small amounts of endogenous peroxidase (Fig 5A), whereas many contained no endogenous peroxidase at all All cell membranes were smooth

In vitro

Essentially, there was no difference in the localization of HRP *in vitro* as compared to the *in vivo* experiments

At 10 minutes after incubation HRP was seen attached to the cell membrane and in the phagolysosomes After this period only a few cells were observed which still had HRP attached to the cell membrane A detail of such a cell is shown in Fig 4, which clearly illustrates the manner in which HRP is localized on the cell surface and in the phagolysosomes of guinea pig macrophages which had been incubated with HRP for 60 minutes However, it should be emphasized that the majority of macrophages from both species of animals had no reaction for HRP on the cell surface

Control cells again showed very little reaction for endogenous peroxidase, although occasionally cells were found which contained HRP in dense granules (Fig 6)

Since the results on the localization of HRP in mouse and guinea pig macrophages were identical for short time intervals, we used only guinea pigs for a 24 hour experiment Guinea pigs were injected i p with 1 mg or 10 mg HRP in saline *in vivo* Many of the macrophages, at this time interval, were in a stage of dissolution and contained large vacuoles, but in many cases the cells did not appear to differ significantly from the controls HRP could still be discerned in well preserved macrophages but the reaction was weaker suggesting that the peroxidase had been degraded Some of the cells which con

Fig 3 High magnification of the cytoplasm and cell membrane of a mouse macrophage which has ingested HRP harvested at 30 minutes after injection The mouse was injected with 10 mg HRP i p Note here that the HRP reaction product is present in the phagolysosomes (P) but that the cell membrane (CM) appears to be rough and to stain slightly for peroxidase × 93 000

Fig 4 High magnification of the cytoplasm and cell membrane of a guinea pig macrophage which has been incubated with HRP for 60 minutes *in vitro* Deposits of HRP reaction product are seen in the phagolysosomes (P) and there is a reaction for peroxidase on the cell membrane (CM) × 93 000

1µm

5'

1µm

5B



Fig 6 ... genous ... ours as control (see text) There is little endo-

tained I ... also contained cell debris, and in some cases whole polymorphonuclear cells were seen inside vacuoles, indicating phagocytic activity

A few cells were observed which appeared to have HRP attached to the cell surface, unevenly distributed Experiments using radioactively labelled peroxidase were therefore carried out in order to ascertain whether antigen was in fact present on the cell membrane

Fig 5 A and B A macrophage and an eosinophilic leucocyte from a pool of cells from control mice Mice were injected with saline and the cells were harvested after one hour Note that there is very little endogenous peroxidase present in the macrophage whereas the granules of the eosinophil stained intensively for peroxidase The two cells were together in the same se t on which in this case was stained with magnesiu n uranylacetate and lead citrate × 16 000

Localization of ^{125}I-labelled Peroxidase

One hour after incubation with ^{125}I-HRP about 0 1 per cent HRP was associated with peritoneal exudate cells After a further incubation for 3 hours, in medium devoid of antigen, about 30 per cent of the radioactivity initially ingested was still associated with the cells

Autoradiographs of cell smears were examined in the light microscope Sections 1-2 μ thick, toluidine blue stained, were prepared from Vestopal embedded material and these were also studied in the light microscope Approximately 5000 cells on the cell smears were counted and about 6000 sectioned cells from the embedded material were also counted In each case 14-15 per cent of the total cells were found to be highly radioactive Cells were not included which showed 20 grains or less, the grains were unevenly distributed over the cell and varied greatly in num-



Fig 7 Electron microscope autoradiograph of a mouse macrophage incubated with 1 mg ^{125}I HRP type VI Silver grains can be seen overlying the dense phagolysosomes (P) which are also stained for peroxidase (see text) The framed part of the cell is illustrated in Fig 8 × 16,000

ber from cell to cell It was difficult to be certain whether the grains were on the cell surface or intracellularly situated

The cell illustrated in Fig 7, which was examined in the electron microscope, was highly radioactive and was typical in that no grains were seen lying free in the cytoplasm A detail of this cell (Fig 8) shows that the grains do lie over phagolysosomes, but that there are phagolysosomes which stain for peroxidase which do not contain silver grains

Of the sectioned cells (about 400) examined in the electron microscope, two or three showed grains over the nucleus but more noteworthy was the paucity of intact cells which definitely had grains on or near the periphery of the cell A detail of a part of such a cell is illustrated in Fig 9, and it is evident that some radioactive grains are in fact on the cell surface However, it should be mentioned that the resolving power of the method, under our experimental conditions, is probably as high as 0 2 μ (for references, see *Jacob* 1971) Thus, some grains on the cell surface might originate from neighbouring phagolysosomes

Fig 8 High magnification of the framed part of the cell shown in Fig 7 The silver grains (arrows) due to ^{125}I-HRP lie over the phagolysosomes (P) which are also stained for peroxidase Some of the phagolysosomes although stained for peroxidase do not contain silver grains Note the absence of grains in other parts of the cytoplasm Silver grains which lie beside the phagolysosomes could in fact emerge from these organelles (see text) × 93,000

Fig 9 High magnification of a mouse macrophage incubated with 1 mg ^{125}I HRP type VI Silver grains can be seen overlying the dense phagolysosomes (P) which are also stained for peroxidase Some silver grains on the plasma membrane (CM) of the cell are also present × 93,000

P

8 100nm

P P

CM

DISCUSSION

The present studies on the localization of HRP in mouse and guinea pig macrophages, *in vivo* and *in vitro*, indicate that the HRP is chiefly sequestered in phagolysosomes as early as 10 minutes after injection *in vivo* or incubation at 37° C *in vitro* HRP was still present in the phagolysosomes 24 hours after injection *in vitro*, although the staining was weaker suggesting that the peroxidase had been degraded This is consistent with what is known about the kinetics of degradation of ^{125}I HRP *in vivo* (*Rhodes*, unpublished observations) and *in vitro* (*Steinman* & *Cohn* 1972)

HRP was found in the endoplasmic reticulum (ER) in only a very few cells, whereas *Catanzaro et al* (1969) reported the presence of HRP in both ER and the perinuclear space However, *Cotran* & *Litt* (1970) have pointed out that endogenous peroxidase is stained under the experimental conditions employed by *Catanzaro et al* Our experiments are in accordance with these observations, since we observed very little endogenous peroxidase in macrophages In contrast the granules of eosinophilic cells, which contain large quantities of endogenous peroxidase, were highly stained under our experimental conditions (Fig 5 B) Our results are thus in complete agreement with those of *Cotran* & *Litt* (1970) in that we were unable to detect exogenous peroxidase in the ER or the perinuclear space

On the other hand we had the impression that a few macrophages still had HRP attached to the cell membrane 24 hours after injection *in vivo* the HRP was distributed more unevenly along the plasmalemma than was the case up to one hour after injection or incubation at 37° C

In an attempt to elucidate this point ^{125}I HRP was incubated with macrophages *in vitro* These experiments demonstrated that about 30 per cent of the ^{125}I HRP ingested by the cells after incubation for one hour persisted when the cells were re incubated in medium devoid of antigen for a further 3 hours Ultrastructural studies indicated that the vast majority of the radioactivity was localized in phagolysosomes This finding is in agreement with that of *Steinman* & *Cohn* (1972) and *Anteunis et al* (1972) although the latter authors did not employ radioactive HRP in addition to cytochemical staining for HRP

Recently *Bona et al* (1972) demonstrated that ^{125}I Maia squinado haemocyanin (MSH) and ^{14}C endotoxin ingested by guinea pig macrophages were localized on the cell surface or in the cytoplasm (free or in vacuoles) These authors incubated macrophages *in vitro* for one hour at 37° C with 100 μg ^{125}I MSH or 150 μg ^{14}C-endotoxin and they found that many macrophages contained silver grains on the cell surface, whereas we used 1 mg ^{125}I HRP and observed only 2 3 cells with HRP on the surface One explanation for the difference in results could be due to the fact that peroxidase is ingested by macrophages to a lesser extent than MSH at 1-4 hours after injection (*Rhodes*, unpublished observations) Therefore, much larger quantities of ^{125}I HRP should probably be used in order to obtain the sort of results reported by *Bona et al* (1972)

Antigen that is attached to the cell membrane has been shown to be immunogenic (*Unanue* & *Askonas* 1968a, *Unanue et al* 1969, *Unanue* & *Cerottini* 1970, *Schmidtke* & *Unanue* 1971), and the immunogenic portion of the molecule can be released by EDTA (*Askonas* & *Jaroskova* 1969) or trypsin (*Unanue* & *Cerottini* 1970)

Schmidtke & *Unanue* (1972) incubated mouse macrophages with 10 mg human serum albumin (HSA) in order to test the immunogenicity of the macrophage bound HSA in recipient mice (primary response) *Mouse* macrophages had also to be incubated with 10 mg HRP before a primary response could be induced in recipient mice to macrophage bound HRP (*Rhodes* unpublished observations) In contrast only 100 μg MSH (*Unanue* & *Askonas* 1968b) was necessary for incubation with macrophages in order to test the immunogenicity of macrophage bound MSH in recipient mice on the other hand th

amount of MSH bound to the cells did not induce a primary response, but primed the mice for a secondary response However, all these observations lend support to the idea that larger amounts of soluble antigens should be incubated with macrophages in order to detect them on the cell surface

Undegraded antigen is considered to be the immunogen, and *Askonas et al* (1968) suggested that antigen might be accessible to the lymphocytes by virtue of its localization on the surface of macrophages Recent experiments (*Bona et al* 1972) indicate that radioactive antigens (MSH and endotoxin) might be transferred from macrophage to lymphocyte and that transferred radioactivity resides partly in the nucleus However, it has still to be proven that such a transfer is tantamount with antibody synthesis

Our results appear to be in accordance with those of others (*Anteunis et al* 1972, *Steinman* & *Cohn* 1972) in that HRP is not localized on the surface of macrophages when tested for cytochemically A few cells containing HRP on the surface were observed when radioactively labelled HRP was used for identification However we feel that caution should be exercised in the interpretation of these results since the localization of antigens on the surface of macrophages may to a large extent be dependent on the dosage and type of antigen employed

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DISINTEGRATION OF SINGLE CELLS IN SUSPENSION

Isolation of rabbit polymorphonuclear leucocyte granules

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Disintegration of rabbit polymorphonuclear leucocytes in suspension is achieved by extrusion under controlled pressure The operating pressure is the key factor influencing the degree of disintegration The number of cells disintegrated increases with higher pressure At 35 kp/cm², 80 90 per cent of the cells are disintegrated The disintegrated material was subsequently analysed by following the subcellular distribution of leucocyte A and B granule marker enzymes The analysis reveals that although 80 90 per cent of the cells are disintegrated, a high degree of subcellular integrity remains Leucocyte A and B granules abundantly present in the disintegrated material, were separated from soluble components by zonal centrifugation Extrusion under controlled pressure as a cell disintegration method is discussed, particularly with reference to the study of polymorphonuclear leucocyte structure and function

The problem of preparing mammalian cell homogenates for the subsequent isolation of subcellular components is well recognized Single cells in suspension seem particularly difficult to homogenize The efficiency of instruments in general use, i e Potter Elvehjem and Dounce type of homogenizers by which to break a sufficient proportion of the cells within a reasonable time is low and diminishes markedly with greater volumes A prolonged homogenization time means that components liberated early in the procedure are exposed to disrupting forces and enzymatic activity of the liberated cell constituents for a longer time The requirement for substantial disruption of cells is then always in conflict with the requirement for preservation of the integrity of labile subcellular components such as lysosomes A method that exposes each cell to the disintegrating activity only once would overcome some of the difficulties This communication deals with such a method

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MATERIALS AND METHODS

Cells

Polymorphonuclear leucocytes (PMN cells) were collected from the peritoneal cavity of rabbits after injecting 100 ml 0 1 per cent glycogen (Nutritional Biochemical Corporation Cleveland Ohio U S A) solution as described elsewhere (19) Care was taken to avoid red cell contamination The cells were washed twice in Krebs Ringer phosphate buffer (pH 7 2) containing 10 mM glucose To lyse red cells the pellet was suspended in 6 ml cold water After 10 20 sec, 2 ml of 3 4 per cent phosphate buffered saline was added and the white cells were harvested by centrifugation (200 × g 10 min) Finally, the leucocytes were suspended in 0 25 M sucrose tris (10 mM) EDTA (1 mM) medium, pH 7 2, to a volume of 25-40 ml (10^7 cells/ml) The cells were counted in a Burker counting chamber after staining with Turk's reagent Viability, as tested with trypan blue exclusion, always exceeded 95 per cent.

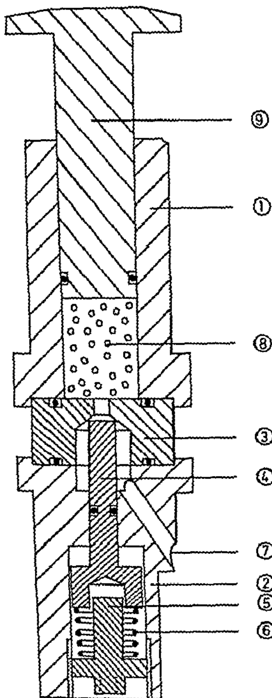


Fig 1 LoX press as m od f ed for d s nt grat on of cells in suspension 1 P ure yl nde 2 Recept acle cylinder 3 D sk es le 5 Scr w to adjust pressure on pestle fr Sp] 7 Outlet 8 Suspension to beco which pressure is appl

Apparatus

The cells were disintegrated by means of a modified LoX press (*Edebo et al* to be published) This device was originally designed for disintegration of parenchymatous tissue by extrusion under controlled pressure The modified LoX press (Fig 1) permits treatment of cells in suspension As shown in Figure 1, the press consists of two main blocks (1, 2) These are held together by a threaded ring, not shown in the figure The upper block contains an axial cylindrical chamber, separated from the lower block by a disk (3) This is provided with a conical hole the diameter of which at the top is smaller than the diameter of the pestle (4) A pressure, which can be continuously adjusted by the screw (5) which compresses the spiral spring (6), is applied on the pestle from below This arrangement seals the outlet (7) from the upper chamber containing the sample (8) as long as the pressure in the chamber does not exceed the counter pressure A manually operated hydraulic jack is applied to achieve such a pressure on the piston (9) that the cell suspension (8) is extruded The extrusion pressure is registered with a Bourdon pressure gauge

Evaluation of the Disintegration

Assessment of the disintegration achieved was performed by counting the number of apparently intact cells in a Burker chamber before and after treatment Cell breakage was also registered by the use of an electronic particle-counter (Coulter Counter Model F, aperture diameter 100 μm) The dials of the apparatus were set as follows B = 2 D = 8 and T = variable and calibrated with latex spheres 9 8 μm and 18 8 μm in diameter The threshold value T = 100 corresponded to a particle volume of 305 μm^3 The extrusion pressure was varied and the number of counts at various thresholds (T) used to calculate the number of particles of different size

For the highest pressure in use the result of LoX press disintegration was biochemically characterized by following the distribution of granule marker enzymes in subcellular fractions In order to avoid enzyme contamination from erythrocytes, particular precautions were taken to induce total red cell lysis After pressing the homogenate was centrifuged at 300 g for 5 min and the supernatant carefully pipetted off The pellet was then gently resuspended in sucrose tris EDTA medium using three passes of a loose fitting Teflon® pestle After resuspension centrifugation at 300 g for 5 min sedimented a low g pellet which was resuspended in homogenization medium and designated fraction I The supernatants from these centrifugations were pooled and centrifuged at 27,000 g for 15 min giving a high g pellet which was resuspended in homogenization medium and

designated fraction II The remaining supernatant was designated fraction III All stages in the preparation of the fractions were performed at 4°C by using precooled equipment and reagents

Zonal Centrifugation

I o\ pressed PMN cells were transferred to a Teflon homogenizer After three passes with a fit fitting pestle the homogenate was centrifuged at 300 g for 5 min The resulting supernatant termed the post nuclear supernate was then fractionated by zonal centrifugation Sucrose tris (10 mM) EDTA (1 mM), pH 7 2 of increasing sucrose concentration with densities ranging from 1 07 to 1 28 was loaded into a spinning (2000 rpm) B-XIV Spinco zonal rotor A 20 ml sample was sandwiched between the gradient and a 200 ml overlay of 0 25 M sucrose tris-EDTA medium The rotor was then accelerated to 20 000 rpm and allowed to run for 20 min After decelerating to 2000 rev/min, the gradient containing the frac

in individual fractions of which the sucrose concentration was determined with a refractometer Centrifugation operations were all conducted at 4° C

Enzyme Assays

Myeloperoxidase (MPO) activity was assayed with guaiacol (17) Two reaction mixtures each containing 1 ml of 20 mM guaiacol 2 ml of 100 mM phosphate buffer pH 7 0 and 0 5 ml of an appropriate dilution of the enzyme sample were prepared To one set of this mixture was added 2 ml of 10 mM H_2O to another the blank, was added 2 ml of buffer The increase in absorbance at 470 nm (Δ O D/min) was recorded spectrophotometrically and remained *constant* within the assay time used

Lysozyme was assayed by its lytic action on *Micrococcus lysodeikticus* cells (13) A substrate suspension was prepared by adding lyophilized *M lysodeikticus* cells (Miles 83) to phosphate (0 1M) EDTA (1 mM buffer pH 6 2 to give an optical density of 0 30 at 450 nm One tenth ml of an appropriate dilution of the enzyme sample was added to 3 ml substrate suspension and the linear clearance rate at 450 nm (Δ O D/min) regarded as lysozyme activity

Acid and alkaline phosphatase activities were assayed as outlined by *Barrett* (6) using 10 mM p nitrophenylphosphate as substrate Reactions were carried out at 37° C in acetate buffer pH 5 and glycine NaOH buffer pH 9 8 respectively The reaction system consisted of 0 5 ml 40 mM substrate solution 1 2 ml 0 2 M buffer and 0 3 ml of an appropriate dilution of the enzyme sample

After incubation and addition of 2 ml ice-cold tris (1M) KH_2PO_4 (400 mM), the released p-nitrophenol was read immediately at 420 nm Hydrolytic rates were linear within the incubation times used

β glucuronidase activity was determined as described by *Fishmann & Bernfeld* (12) The hydrolytic rate of 1 mM phenolphtalein mono-β glucuronide in 0 05 M acetate buffer, pH 5 0 at 37° C was taken for β glucuronidase activity

β galactosidase, β glucosidase β fucosidase and N acetyl β glucosaminidase activities were assayed using the corresponding glycoside of o- or p nitrophenol as substrates (6) Incubation mixtures contained 0 5 ml buffer 0 5 ml nitrophenylglycoside substrate and 0 5 ml enzyme sample Citrate-phosphate buffer of suitable acidic pH and appropriate substrate concentrations were chosen according to *Beck & Tappel* (8) The reaction at 37° C was terminated by the addition of 1 5 ml 2 0 M NH_3 and the extinction of the nitrophenyl anion measured as E_{420}

Triton X 100 (0 1 per cent) was used to solubilize particle-bound enzyme Protein was determined according to *Lowry et al* (16)

RESULTS

Quantitation of the Disintegration

When intact suspensions of PMN cells were counted with the electronic particle counter the number of pulses increased as the threshold decreased (Fig 2a) The data indicated a mixed population, the major part with volumes around 230 μm³ (T = 60-90 per cent) being mainly PMN cells the minor part with volumes around 75 μm³ (T = 20 30 per cent) largely being contaminating erythrocytes Pressing the suspension reduced the number of counts the higher the pressure the lower the counts

The percentage of pulses remaining *after* pressing was calculated for the *different* pressures at the different thresholds (Fig 2b) At all threshold settings the fraction of pulses recovered after pressing decreased as the operating pressure increased Down to T = 60 per cent the fraction of pulses measured after one pressing was fairly constant (Fig 2b) At T = 60 *per cent* (B = 2 D = 8) most intact cells were counted (Fig 2a) At lower T values there was a sharp rise in the fraction of pulses counted *after pressing*

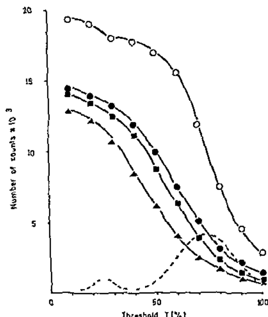


Fig 2a Electronic particle counting of leucocyte suspensions before and after LoX pressing Solid lines show cumulative counts at various thresholds Broken line shows the volume distribution of particles before pressing, trace contamination of red cells appears as increased counts at lower thresholds Operating pressures 25 kp/cm² — ● 30 kp/cm² = ■ 35 kp/cm² = ▲ untreated — ○

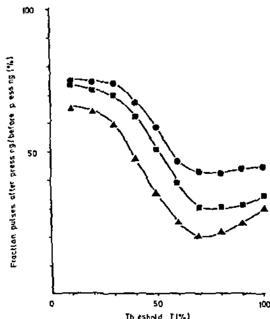


Fig 2b Precentage of pulses recovered after pressing in relation to that recorded before pressing at the same threshold at 25 kp/cm² = ●, 30 kp/cm² = ■ and 35 kp/cm² = ▲

which indicates that particles others than intact cells were counted Therefore T = 60 per cent was selected to measure the number of disrupted cells At 35 kp/cm², 20 per cent of the pulses were recovered and, thus, 80 per cent of the cells were disrupted

The percentage of intact cells, calculated on the basis of counting in a Burker chamber, was generally slightly lower than the percentage calculated on the basis of data obtained with the particle counter Hence, at a pressure of 35 kp/cm², only 10 per cent of the cells were intact as judged microscopically Some cells showed disrupted cell membrane but a rather intact intracellular organization remained

Biochemical Quantitation of Subcellular Integrity

Recent biochemical investigations (3, 4, 21) verified the existance of two main populations of granules in mature rabbit PMN leucocytes, previously known to differ by their size and staining properties (5, 20) The two populations are biochemically distinct in that they possess myeloperoxidase (A particles) and alkaline phosphatase (B particles), respectively Consequently, these enzymes were chosen as granule markers In addition, the distributions of lysozyme and β glucuronidase in subcellular fractions were followed

The distribution of marker enzymes is shown in Figure 3 Although 80 90 per cent of the cells were disrupted, the relative specific activity of myeloperoxidase in fraction III was not more than 0 18, indicating a high degree of A-granule integrity in the homogenate. The same was true of B-granules, the relative specific activity of alkaline phosphatase in fraction III being somewhat higher (0 52)

Lysozyme is reported to have a dual locali-

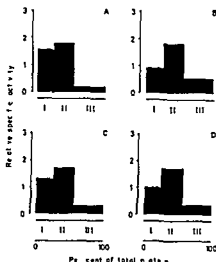


Fig 3 Subcellular integrity after disintegration in the LoX press at 35 kp/cm² as shown by distribution of granule marker enzymes in fractions obtained by differential centrifugation Relative specific activity = per cent of recovered enzyme / per cent of recovered protein Protein recovery over homogenate = 95 105 per cent, enzyme recoveries over homogenate = 95 115 per cent A = myeloperoxidase B = alkaline phosphatase C = β glucuronidase D = lysozyme

zation in mature rabbit polymorphonuclear cells (3, 21) One third of the total activity is carried with the (azurophilic) MPO bearing (A) granule the remainder resides in the (specific) alkaline phosphatase bearing (B) granule In homogenates obtained by LoX press disruption, lysozyme showed a distribution pattern falling roughly between those of myeloperoxidase and alkaline phosphatase lthough it more closely resembled the latter β glucuronidase, which activity largely res des in the myeloperoxidase bearing granule (3) s distributed in a manner closely resembling m eloperoxidase

The high relative pecific activities in fraction I, especially of myeloperoxidase and β glucuronidase, were consistent with the appearance in phase contrast microscopy, i e gentle homogenization of the low g pellet using a few passes with a loose fitting pestle did not release granules from insufficiently disintegrated cells nor dissociate granule aggregates to a desirable extent Better release was accomplished by the use of a fit fitting Teflon pestle in resuspending the 'low g pellet In general, there was a considerable decrease in the relative specific activity of marker enzymes in fraction I with a concomitant increase in fractions II and III (Fig 4) These changes were primarily due to changes in marker enzyme distribution since only minor changes in the distribution of total protein occurred The high activities in fraction II then reflect the large numbers of intact granules sedimented at 27 000 × g *Examination of fraction I in phase-contrast* microscopy showed nuclei together with very few granules and few intact cells Thus this homogenization freed A and B granules from the aggregated structures that were sedimented at 300 g from the original suspension However, the increase in activities in fraction III may suggest that some granules were disrupted and hence marker enzyme released during this process

Zonal Centrifugation

The result of further fractionation of the postnuclear supernate using zonal centrifugation in a sucrose gradient is shown in Fig 5

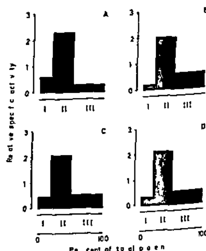


Fig 4 Distribution of granule marker enzymes in subcellular fraction obtained by differential centrifugation The suspension was pressed at 35 kp/cm² A fit fitting Teflon pestle was used to resuspend the material pelleted by the initial centrifugation A = myeloperoxidase B = alkaline phosphatase C = β glucuronidase D = lysozyme

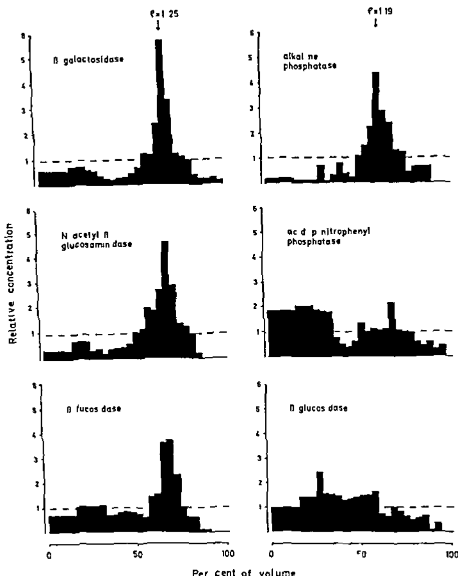


Fig 5 Zonal centrifugation of the postnuclear supernate from rabbit PMN cell homogenate obtained by LoX press d sintegrat on at 35 kp/cm² Results are represented as distribution histograms as a funct on of the volume collected Radial distance increases from left to right Further details are given in the text

A granule markers such as β galactosidase and N acetyl β glucosaminidase (3) gave si milar sedimentation patterns with coincident major activity peaks The distribution of β fucosidase throughout the gradient resembled that of A granule markers In contrast the peak of alkaline phosphatase activity appe ared at lower sucrose density β glucosidase and prominently, acid p nitrophenylphospha tase activities were spread out towards the lighter end of the gradient

The graphs are normalized distribution histograms as a function of the volume col lected and the ordinate shows activity in frac tion relative to a calculated activity corres ponding to uniform distribution throughout

the gradient Again the low activities of granule marker enzymes in the lighter part of the gradient together with the quantitatively evident particle enrichment in denser sucrose emphasize the gentleness of LoX press disintegration

The distribution of acid p nitrophenylphosphatase activity was consistent with reports on its non lysosomal localization in various tissues (7) one of which is rabbit PMN leucocytes (3, 21) The question whether an analogous situation exists for β glucosidase activity may need further analytical experimentation before being discussed

DISCUSSION

The study of subcellular components is intimately tied to the preparative techniques available and the application of different separation techniques to cell homogenates has gained considerable interest However the primary problem is the choice of method by which to release in suitable yields the subcellular components in a state resembling their native condition Ideally such methods should give homogenates consisting solely of released but intact and functioning cell organelles In practice however, it is evident that no homogenization procedure will fulfil this ideal most homogenates as presently prepared contain mixtures of intact cells cell ga lles and other cell components in various stages of disintegration In general homogenizat on must be performed as gently as possible to minimize physical damage to the subcellular components In most cases homogenizers with a rotating pestle as first devised by *Potter* & *Elvehjem* (18) or that of *Dounce et al* (10) are considered to provide sufficient shearing forces to break cells without excessive damage to subcellular components

The wide variations among different tissues and cells both in the resistance to mechanical stress and in the fragility of subcellular components may make the homogenization of each individual tissue a separate problem Homogenization of cell suspensions in conventional pestle homogenizers has proved difficult since with tube pestle clearances sufficient to avoid damage to subcellular elements free cells are able to squeeze past without disruption Thus special means have beeen used to disrupt single cells in suspension *Cohn* & *Hirsch* (9) used repeated forceful pipetting to disrupt polymorphonuclear cells from rabbit peritoneal exudates The granules of these cells appear to be especially fragile (14) *Archer* & *Hirsch* (1) ruptured rat and horse eosinophils by passage through a fine mesh screen under negative pressure. Single cells in suspension have also been homogenized by means of sudden release of gas pressure (2 15)

Whether considered from the preparative or analytical points of view it is important whatever device is used to ensure adequate reproducibility Most disintegration methods are influenced by a number of parameters not amenable to operator control In contrast LoX press disintegration of suspended cells is controlled by adjusting the counterpressure on the pestle (Fig 1 4) and forcing the piston (Fig 1 9) down with such a speed that a constant pressure is maintained under the extrusion of the material When this procedure is adhered to the disintegration is readily reproducible

Cell disintegration is usually evaluated by morphological and biochemical criteria Microscopic examination may be used to count the number of morphologically intact cells and to disclose the presence of aggregation or other secondary phenomena to estimate the degree of structural damage to cells and organelles The successful use of an electron c particle counter for quantitation of yeast cell disintegration (*Magnusson* & *Edebo* to be published) encouraged its use in quantitat ng PMN cell disintegration As judged by microscopic examination and particle counting LoX press disintegration produced extensive PMN cell disruption without the aggregat on of subcellular organelles that may result from prolonged disintegration with a teflon pestle and glass tube (21) Granule aggregates present in homogenates obtained by LoX press disruption were easily dissociated and were

thus the result of insufficient cytoplasmic disintegration rather than incapability of free granules to remain fully dispersed

In further assessing the quality of a homogenate, biochemical criteria allow a quantitative evaluation of the subcellular integrity The sedimentability of lysosomal enzymes depends on the integrity of the lysosomal membrane, this is one of the fundamental elements of the biochemical concept of lysosomes (11) Although all PMN cell granules may not qualify as true lysosomes, the structure linked latency measured as sedimentability of granule marker enzyme activity is a property that can be used to measure granule integrity

Disintegration in the LoX-press followed by differential centrifugation and quantitative determination of marker enzyme activity in the different fractions presented data (Figs 3-4) which agree well with the postulate that cells exposed to a focused mechanical stress of adjusted size release their constituent organelles in a highly organized state Thus, LoX pressed rabbit PMN cells seem to constitute a proper starting material for further granule fractionation That such fractionation is feasible appears from the resolution by zonal centrifugation of marker enzymes for different kinds of granules (Fig 5) Furthermore, the reproducibility of the results indicate that the proneness to particle-association of a marker enzyme after exposure to a fixed extrusion pressure can be used as a mechanical characteristic of the respective organelle

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INFLUENCE OF THE CELL SURFACE LIPOPOLYSACCHARIDE STRUCTURE OF *SALMONELLA TYPHIMURIUM* ON RESISTANCE TO INTRACELLULAR BACTERICIDAL SYSTEMS

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The virulent *Salmonella typhimurium* 395 MS and R mutants derived from it that have deficiencies in their cell wall lipopolysaccharide (LPS) were examined for sensitivity to different bactericidal systems operating within the polymorphonuclear leucocyte (1) the lysosomal granule fraction (8200 × g), (2) the myeloperoxidase hydrogen peroxide halide system and (3) the D amino acid oxidase linked system The S strain was most resistant to all the systems investigated Synthesis and incorporation of complete S like LPS by growing the uridine-diphosphate galactose-4 epimeraseless mutant LT2 M1 in galactose supplemented medium enhanced the resistance to the MPO mediated system as the amount of LPS incorporated increased Among R mutants, bacteria possessing complete or nearly complete basal core LPS (chemotype Ra Rb_2) were less sensitive to the myeloperoxidase-mediated system than were mutants more deficient in their cell wall LPS D amino acid oxidase, in combination with D alanine and a myeloperoxidase-containing leucocyte extract, produced a bactericidal system efficient even on the S strain The very deficient Rd mutant R10, but not the S strain was sensitive to the activity of D amino acid oxidase even without the addition of D amino acids, indicating that the bacteria can serve as an enzyme substrate These results demonstrate the decisive influence of the bacterial cell surface structure on the efficiency of intracellular bactericidal systems on enterobacteria.

The importance of cell surface interactions in the host parasite relationship has been stressed in a number of studies on different biological systems Differences in the cell wall lipopolysaccharide (LPS) of mutants of *Salmonella typhimurium* 395MS (11, 16) confer sufficient surface changes to the bacteria to alter their virulence for mice (6) and their interaction with phagocytes at the engulfment step (28)

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Successful phagocytosis is of crucial importance in the eradication of invading parasites Strictly speaking, host resistance to bacterial infection is ultimately dependent on the efficiency of both the engulfment and the subsequent intracellular processing of the phagocytosed parasites The experiments described in this paper were designed to examine whether differences in bacterial surface structure are reflected as differences in resistance to antibacterial systems operating within the phagocytic cell Interest was focused on the

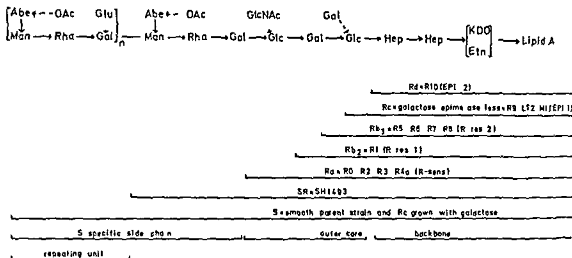


Fig 1 Proposed structures of the LPS of *S typhimurium* The lower part of the figure shows the relation between structure and *chemotype* (*S*, *SR*, Ra Rd), phage pattern (smooth, R sens, etc) and the strains used *in this study* A dotted line indicates that not all the molecules carry the indicated substituent Abbreviations OAc O acetylgroup, Abe, abequose, Man, D mannose Rha L-rhamnose, Glc D glucose, Gal, D galactose GlcNAc, N acetyl D glucosamine, Hep, L-glycero D manno heptose, KDO, 2 keto-3 deoxyoctonate, *Etn*, *ethanolamine*

interaction of bacteria with the leucocyte myeloperoxidase system (14, 26) and the associated D amino acid oxidase linked system (1)

MATERIALS AND METHODS

Bacterial strains The smooth mouse virulent strain *Salmonella typhimurium* 395MS, the rough cell wall deficient mutants derived from it (RO R10) and the uridinediphosphate (UDP) galactose (gal) 4 epimeraseless mutant LT2 M1 from *Salmonella typhimurium* LT2 have been described earlier (6 11 16 17) The semirough mutant SH1493 obtained from *S typhimurium* LT2 was kindly suppl ed by Dr *P H Makela*, Helsinki Finland The proposed LPS structure of the strains is depicted in Fig 1

Cultivation All strains were kept at 4° C on agar slants before use Their phage patterns were regularly checked From an overnight culture in nutrient broth (D fco) 1 ml was transferred into 9 ml fresh medium and incubated for another 4 h at 37° C on a rotary shaker The bacteria were harvested by centrifugation (6000 × g) and washed twice in phosphate buffered saline solution pH 7 2 (PBS) The concentration of bacteria was estimated with a Turner spectrophotometer (650 nm) The bacteria were finally diluted to a concentration of 10^{7} bacteria/ml in citratephosphate buffer, pH 5 5 for use

The UDP gal 4 epimeraseless mutant LT2 M1 was cultivated according to *Lindberg et al* (17) The mutant was grown *in glucose medium to a* concentration of 2 × 10^{8} bacteria/ml Then D galactose was added to give a final concentration of 2 g/l During the subsequent incubation samples were taken at intervals (0 10, 20, 40 60 and 180 min) These were immediately cooled centrifuged (6000 × g, 10 min) and washed twice in cold PBS The harvested cells were immediately exposed to the myeloperoxidase hydrogen peroxide iodide bactericidal system

Polymorphonuclear (PMN) leucocytes Rabbit peritoneal PMN cells were obtained as previously described (28) Care was taken to avoid red cell contamination After collection the cells were washed twice in Krebs Ringer phosphate buffer (pH 7 2) To lyse contaminating erythrocytes the pellet was briefly suspended in distilled water Finally the leucocytes were suspended in 0 25 M sucrose tris (10mM) EDTA (1mM) medium pH 7 2 for homogenization

PMN cell granule fraction was prepared as described by *Friedberg & Shilo* (7) PMN cells were homogenized in a chilled Dounce type homogenizer with a Teflon pestle and then fractionated as described by *Cohn & Hirsch* (2) The granule fraction (8200 × g) was lysed by freezing and thawing five times The material obtained is referred to as the lysed granule fraction (LG) Protein was determined according to *Lowry et al* (18)

Myeloperoxidase (MPO) was prepared from postnuclear supernates of PMN cell homogenates obtained by LoX press disruption (*Tagesson et al* to be published) Intact cells nuclei and cellular debris in the homogenate were pelleted by centri

fugation at 300 × g for 5 min and discarded The supernate, called the postnuclear supernate, was centrifuged again at 27,000 × g for 20 min and the resulting pellet suspended in distilled water before being subjected to five freeze thawing cycles MPO activity was determined with guaiacol (19) as described elsewhere (*Tagesson et al*, to be published) One unit (U) of MPO activity was taken as the amount of enzyme giving a ΔOD_{470} nm of 0 001/min in a reaction volume of 5 5 ml

D amino acid oxidase (DAAO) Purified hog kidney DAAO was purchased from Worthington Biochemical Corporation, Freehold, New Jersey, U S A Enzyme activity was determined with 3 methyl 2 benzothiazolone hydrazone hydrochloride (MBTH, Aldrich Chemical Company, Inc, Milwaukee, Wisconsin, U S A), as described by *Soda* (27) Reactions were carried out in tris (hydroxymethyl) aminomethane buffer, pH 8 3, at 37° C One unit (U) of DAAO activity was defined as the amount that oxidatively deaminates 12 5 mM D alanine at the rate of 1 mμmol/min in the presence of 80 μm FAD (flavin adenine dinucleotide)

Bactericidal assays were carried out in citrate phosphate buffer, pH 5 5, in siliconized tubes at 37° C in a rotary shaker The initial reaction volume was 1 ml unless otherwise stated Samples (0 1 ml) were withdrawn at intervals and the viable cell count determined by plating onto nutrient agar after suitable dilutions

RESULTS

Sensitivity of Salmonella typhimurium *395MS, R0 and R10 to the bactericidal activity of the lysed granule fraction (LG)* The bactericidal effect of the LG on *Salmonella typhimurium* 395MS and two R-mutants derived from it (R0 and R10) is shown in Fig 2 The results are consistent with those obtained by others (7), i e R0, though lacking S-specific side chains on its cell wall LPS (chemotype Ra), showed resistance similar to that of the parent smooth strain, while R10, which in addition to S-specific side chains also lacks to the outer core structure of the LPS (chemotype Rd), was less resistant

Sensitivity of Salmonella typhimurium *of different cell wall compositions to the bactericidal activity of a myeloperoxidase mediated antimicrobial system* It has been proposed that one of the main bactericidal mechanisms within the leucocyte is dependent on the MPO mediated breakdown of H O (14 26) In the leucocyte, H O is supplied metabolically In vitro, the MPO H O system might be mimicked by adding H_2O to a lysed lysosomal fraction Iodide ions further enhance the bactericidal effect (13) To establish the MPO H_2O KI system an improved method for disintegration of leucocytes was used (*Tagesson et al*, to be published) A MPO mediated system was made up from the freeze-thawed 27,000 × g pellet deposited from a postnuclear supernate Since PMN cell granules contain a number of antibacterial factors along with various hydrolytic enzymes, the concentration of the PMN cell extract was adjusted in such a way that it was non-bacterici-

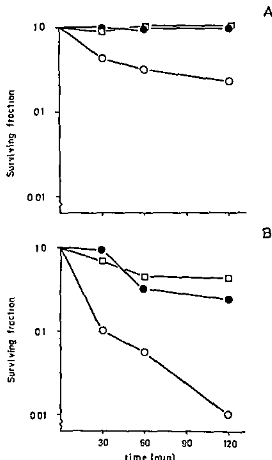


Fig 2 Bactericidal effect of the lysed granule fraction (LG) on *Salmonella typhimurium* 395MS ● R0 = □ and R10 = ○ The initial number of bacteria was 5 × 10^7 cells in a reaction volume of 3 ml pH 6 0, containing 20 μg LG protein (A) or 200 μg LG protein (B) per ml

of the PMN lysosomes have, for the most part concentrated on the properties of the lysosomes themselves Little attention has been paid to the interaction of lysosomes with the bacterial surface *Prixota* (23) demonstrated that rough strains of *E coli* and *S typhimurium* were more sensitive than smooth strains to the bactericidal activity of an extract from rabbit polymorphonuclear cells Using cell wall mutants of Salmonella with known deficiencies in the sequence of polysaccharide in their cell walls *Friedberg* & *Shilo* were able to show that the complete LPS basal core was essential for resistance to the lysed lysosomal fraction from guinea pig PMN cells (7) The present study agrees quantitatively with their results and confirms the proposed relationship between bacterial cell wall LPS structure and resistance to the bactericidal activity of PMN lysosomal fraction At a concentration of 20 μg LG protein per ml, 30 per cent of the Rd mutant R10 survived after one hour, but all of the S bacteria (MS) survived as did the Ra mutant (R0) At 200 μg per ml, 6 per cent of R10 and 30 per cent of the MS and Ra bacteria survived (Fig 2) It thus appears that S specific side chains do not contribute markedly to the resistance to the LG fraction although defects in the core structure render the bacteria more sensitive

The dramatic increase in hexose monophosphate shunt activity following particle uptake by leucocyte leads to an increased production of NADPH which is oxidized by glutathione reductase (29) and in the presence of molecular oxygen by NADPH oxidoreductase (12 24) to yield H_2O_2 This H_2O_2 can be attacked by the MPO present in leucocyte granules yielding a bactericidal product the lethal activity of which is enhanced in the presence of halide ions (13) The addition of nonbactericidal concentrations H_2O_2 and iodide to granule extracts has been used as a substitute for the conditions assumed to exist in vivo Peroxidase activity was demonstrated in our MPO preparation by the guaiacol method which shows that the preconditions for generating bactericidal activity via the MPO H_2O_2 iodide system were fulfilled

The addition of nonbactericidal concentrations of H_2O_2 and iodide to a likewise non bactericidal MPO preparation produced an efficient bactericidal system (Fig 3) The preparation, which contained approximately 10 μg/ml protein, showed a bactericidal activity on R0 and R10 much stronger than produced by 200 μg/ml of an LG fraction without H_2O_2 and iodide Since killing did not occur whether H_2O_2, iodide or the MPO preparation alone were used, this enhancement is considered to be the result of the MPO H_2O_2 halide system The bactericidal effect on MS was negligible If the mutants R0 R10 were tested in the same system those with the most deficient LPS (chemotypes Rb_3 Rd) were more sensitive than those with the less deficient LPS (chemotype Ra Rb_2) These results are similar to those presented by *Friedberg* & *Shilo* (7) which indicated that the complete LPS basal core was essential for resistance to the lysed granule fraction Our data suggest that the two D galactosyl units in the core contributed the most to the increase in resistance to the *MPO H_2O_2 halide* system The presence of S specific LPS further increased the resistance, the degree of increase being dependent on the quantity of S specific repeating units (Figs 3 and 4) The repeating units had a higher protective effect in polymeric than in monomeric form since the SR mutant was fairly sensitive while MS and LT2 M1 grown in the presence of D galactose were resistant (Figs 3 and 4) These data suggest that the presence of S-specific side chains on the bacterial cell surface does contribute toward resistance to intracellular degradation and in fact, loss of S-specific side chains causes a drastic reduction in bacterial resistance to intracellular killing

These observations are consonant with an enhanced resistance to the bactericidal action of antibody and complement when S specific side chains are present on the cell surface (4 15 21) Furthermore, *Dlabač* (5) showed that an Rc mutant (UDP gal-4 epimerase

less) grown in galactose supplemented medium acquired resistance to the bactericidal action of precolostral piglet serum In the present investigation, the same mutant showed increased resistance to the MPO mediated bactericidal system (Fig 4) The acquired resistance, however, did not develop until 120 180 min after the addition of galactose, in spite of the fact that synthesis of complete S type LPS takes place, within 10 min (17) Apparently, large numbers of S specific determinants are necessary to protect the cell, since the number of S specific side chains present on the cell is ten times higher after 180 min than after 10 min (17)

Another source of H_2O might be supplied by oxidative deamination mediated by D amino acid oxidase This activity is present in PMN leucocytes and is suggested to constitute a biochemically specific system recognizing the 'foreigness" of phagocytosed microorganisms *Cline* & *Lehrer* (1) reported that bacterial cells could serve as substrate for D amino acid oxidase, yielding H_2O Such cell wall D amino acids as are not in peptide linkage (25) would be vulnerable to attack by the oxidase The extent of D amino acid oxidase mediated production of H O should then depend on the quantity of enzyme available and its access to adequate substrate Such access and therefore also the efficiency of the bactericidal system depends upon the surface structure of the bacteria With exogenous source of D amino acid, the DAAO linked bactericidal system was very efficient both on MS and R10 and was capable of causing more than 99 9 per cent killing of either of the bacteria Against R10 the bactericidal effect was decreased but still significant after omission of exogenous D alanine indicating that the enzyme substrate was available in these bacteria, presumably as a consequence of the deficient surface LPS Omission of exogenous D alanine completely abolished the bactericidal effect on MS indicating that DAAO substrate was not accessible in lethal amounts

The target for the bactericidal action of serum as well as that for enzyme linked systems seems to be buried in the outer envelope of the bacterial cell (22) Complete S-type LPS may sterically cover or block both DAAO generation of bactericidal compounds and the target for all the different bactericidal compounds which exist in the cell and so frustrate the bactericidal effect In addition, the presence of complete S antigen is known to change the overall physico-chemical properties of the cell surface (*Stendahl et al* to be published) which in turn could make the ingested bacteria less liable to evoke an intra cellular biochemical activation Of particular interest in this respect is the report that phagocytosis of a virulent *S typhi* strain by polymorphonuclear leucocytes is not accompanied by an enhanced oxygen consumption while ingestion of a less virulent strain is associated with the classical post phagocytic increase in oxygen uptake (20)

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PHAGOCYTOSIS OF *SALMONELLA TYPHIMURIUM* 395 MR10 BY RABBIT POLYMORPHONUCLEAR LEUCOCYTES: FORMATION OF PHAGOLYSOSOMES DEMONSTRATED BY ZONAL SEPARATION

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After allowing phagocytic uptake of bacteria rabbit polymorphonuclear leucocytes were disintegrated and fractionated by zonal centrifugation Two different subcellular particles were obtained containing high relative activities of lysosomal marker enzymes One particle displayed the sedimenting characteristics of lysosomes the other sedimented with radio actively labelled bacteria subjected to phagocytosis The properties of the latter particles and the conditions concerning their formation were those expected of phagolysosomes The phagolysosomes showed a peak at a density = 1 19 whereas both lysosomes and non phagocytosed bacteria were recovered at a density = 1 25 The results are discussed in relation to the analysis of host parasite interaction at a subcellular level

The intracellular events following phagocytic uptake of bacteria are of fundamental interest in studies of the host parasite relationship *Hirsch & Cohn* (6) described degranulation of rabbit polymorphonuclear leucocytes following uptake of bacteria Microcinematographic studies enabled *Hirsch* (4) to discern a series of discrete intracellular events during the interaction after the initial formation of a phagosome, granules approached this structure and finally coalesced with it Subsequent studies using electron microscopy (18) revealed that the granules merge with the phagosome through a process of membrane fusion This is followed by a discharge of the granule contents into the phagosome, which is thought to be a mechanism ensuring the localization of bactericidal agents and enzymes necessary for the breakdown of bacteria in a discrete cell compartment the phagolysosome The phagolysosome plays apparently a key role in phagocyte bacterium interactions The process of its formation and the analysis of the bactericidal and digestive events taking place within it has almost exclusively been characterized by morphological criteria (1, 5) Further analysis of these events is intimately dependent on the separation of phagolysosomes from other subcellular elements The experiments described in this communication were designed to demonstrate by means of zonal separation the formation of phagolysosomes in actively phagocytosing polymorphonuclear leucocytes

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MATERIALS AND METHODS

Bacterial strain The rough cell wall deficient mutant R10 (chemotype Rd) derived from *Salmonella typhimurium* 395 MS has been described earlier (7 8)

Cultivation The strain was kept at 4° C on agar slants Bacteria were inoculated into 10 ml nutrient broth (Difco) and incubated at 37° C for 18 h Then 1 ml of the culture was transferred into 9 ml fresh medium and incubated for another 4 hours at 37° C on a rotary shaker The bacteria were harvested by centrifugation (6000 × g) and washed twice in phosphate buffered saline solution pH 7 2 (PBS) Before labelling the bacteria were heat killed at 56° C for 1 h and washed twice in PBS

Labeling Heat killed bacteria were labelled with ^{51}Cr and ^{125}I as described elsewhere (*Stendahl et al* 1973 in press)

Polymorphonuclear leucocytes (PMN cells) Rabbit peritoneal PMN cells were obtained as described elsewhere (11) Care was taken to avoid red cell contamination After collection the cells were washed twice in Krebs Ringer phosphate buffer pH 7 2 (KRB) To lyse contaminating erythrocytes the pellet was exposed to distilled water Following red cell lysis leucocytes were harvested by centrifugation (200 × g 10 min) and washed once in KRB

Phagocytosis system PMN cell suspensions were divided into two equal portions One portion was exposed to bacteria and allowed to phagocytose The experimental mixture consisted of 100 ml Krebs-Ringer phosphate buffered solution pH 7 2 (KRB) containing 10 mM glucose 5 per cent normal rabbit serum 3 × 10^6 PMN cells per ml and 3 × 10^7 ^{51}Cr labelled R10 bacteria per ml The second portion of the PMN cell suspension served as a control the cells were treated exactly as in the experimental mixture except that bacteria were omitted Both preparations were incubated for 15 min at 37° C and then chilled in an icebath They were then centrifuged at 100 g for 5 min to deposit the PMN cells These were then washed once in KRB at 100 g for 5 min leaving most non phagocytosed bacteria in the supernate Finally the PMN cells were suspended in 0 25 M sucrose tris (10 mM) EDTA(1mM) medium pH 7 2 The two preparations were then pooled into a single suspension (40 ml 2 5 × 10^8 cells per ml) and held at 4° C until further processed

Disintegration The PMN cell were disintegrated by extrusion at 35 kp/cm² in the LoX press (*Tagesson et al* to be published) and further homogenized in a prechilled Teflon homogenizer using three passes with a loose fitting pestle Intact cells nuclei and cellular debris were pelleted by centrifugation at 300 × g for 5 min and the post nuclear supernate carefully pipetted off Before further fractionation 5 × 10^9 ^{125}I labelled R10 bacteria were added to the post nuclear supernate

Zonal centrifugation Sucrose tris (10 mM) EDTA(1 mM) of increasing sucrose concentration with densities ranging from 1 07 to 1 28 was injected into a spinning (2000 rev/min) B XIV Spinco zonal rotor Then the 40 ml sample consisting of the post nuclear supernate with the added ^{125}I labelled R10 bacteria was introduced After adding an overlay of 40 ml homogenization medium the rotor was accelerated to 13 000 × rpm and run at that speed for 13 min After deceleration the gradient containing the fractionated components was displaced from the spinning rotor (2000 rev/min) with 60 per cent (w/w) sucrose The effluent was collected in individual fractions of which the sucrose concentrations were determined in a refractometer All centrifugation operations were conducted at 4° C

Analysis Radioactivity was estimated with an auto-gamma scintillation counter with a NaI-crystal (Packard Instrument Co Downers Grove Ill. USA)

β galactosidase β glucuronidase and N acetyl β glucosaminidase activities were assayed according to standard methods as described elsewhere (*Tagesson et al*, to be published) Lactic dehydrogenase (LDH) was determined by the method of *Morgenstern et al* (10) Triton X 100 (0 1 per cent) was used to release latent enzyme activity in fractions after zonal centrifugation as shown in *Fig 1* In order to release membrane bounded bacteria and enzymes before zonal centrifugation (Fig 2) 0 2 per cent Triton X 100 was used

RESULTS

The distribution of radioactivity throughout the density gradient after zonal centrifugation of the post nuclear supernate *is depicted* in Fig 1 ^{125}I radioactivity as indicator of bacteria not subjected to phagocytosis sedimented as a uniform particle population *in a* Gaussian distribution with its peak in the denser part of the gradient arbitrarily termed the A region (Fig 1A ρ = 1 25) *In contrast* the distribution of ^{51}Cr radioactivity as *indicator* of bacteria subjected to phagocytosis versus density was strikingly *different* and more complex (*Fig 1B*) The bulk of ^{51}Cr radioactivity were banded at lower sucrose density termed the B region (Fig 1B, ρ = 1 19), although some activity appeared together with ^{125}I activity in the A region (*Fig* 1B, ρ = 1 25)

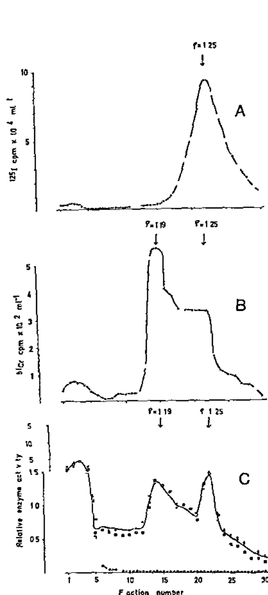


Fig. 1. Resolution of phagocytic markers by zonal separation. For a detailed description of the experimental system see Materials and Methods section. Radial distance increases from left to right. ρ = density of the sucrose gradient. A. Distribution of ^{125}I labelled non phagocytosed bacteria. B. Distribution of ^{51}Cr labelled bacteria exposed to PMN leucocytes. C. Distribution of leucocyte marker enzymes. —— = mean of relative β galactosidase (□), β glucuronidase (●) and N acetyl β glucosaminidase (○) activities. ---- = relative LDH activity (■). Relative enzyme activity = enzyme activity in fraction relative to activity corresponding to uniform distribution throughout the density gradient.

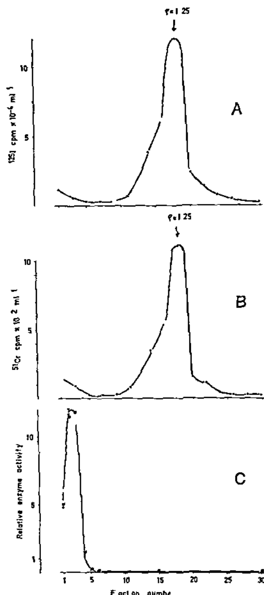


Fig. 2. Effect of Triton X 100 on the resolution of phagocytic markers by zonal separation. For a detailed description of the experimental system, see Materials and Methods section. Results are represented as in Fig. 1. A. Distribution of ^{125}I labelled non phagocytosed bacteria. B. Distribution of ^{51}Cr labelled bacteria exposed to PMN leucocytes. C. Distribution of leucocyte marker enzymes. —— = mean of relative β galactosidase (□), β glucuronidase (●) and N acetyl β glucosaminidase (○) activities. No enzyme activity could be detected beyond fraction 5.

LDH, a cytoplasmic enzyme, did not enter the gradient Similarly, significant amounts of the acid hydrolases analysed, i e β galactosidase, β glucuronidase and N acetyl β glucosaminidase, did not sediment In contrast to LDH, however, the acid hydrolases showed high activities in the A- and B regions Control experiments in which non phagocytosing PMN cells were disintegrated and the post-nuclear supernate sedimented by zonal centrifugation localized the acid hydrolases to the A region under the experimental conditions used The amounts of nonsedimentable enzyme activity was under these conditions small and no enrichment of acid hydrolases was seen in the B region

If Triton X-100 (0 2 per cent) was added to the sample before zonal centrifugation a marked shift in the distribution became manifest (Fig 2) Bacteria exposed to intact leucocytes were distributed essentially the same as bacteria exposed to the post nuclear supernate of a leucocyte homogenate Both bacteria accumulated in the denser part of the gradient (A region), whereas the acid hydrolases did not enter the gradient, suggesting that these enzymes were no longer particle associated

DISCUSSION

The data presented in Fig 1 suggest that, in the system used four different particles were demonstrated Three different particles concur in the A region (ρ − 1 25) two different kinds of nonaffected bacteria represented by ^{51}Cr labelled bacteria left nonaffected by the polymorphonuclear phagocytes (Fig 1B) and ^{125}I labelled bacteria never subjected to phagocytosis (Fig 1A) the same applies to leucocyte lysosomes (Fig 1C, ρ = 1 25) In control experiments bacteria without leucocytic material and lysosomal marker enzymes from a leucocyte post nuclear supernate without bacteria sedimented in the A region Accordingly, when the subcellular structures of the leucocytes were broken down by Triton X 100 all bacteria accumulated in that region (Fig 2A 2B)

The fourth type of particles appearing in the B region after phagocytosis (Fig 1B and 1C, ρ = 1 19), contained both bacteria exposed to phagocytosis and lysosomal enzymes whereas such bacteria that were added after the phagocytic process were not present in this region Since treatment with Triton X 100 upheaved the sedimentation characteristic of the bacteria aquired in the phagocytic process these particles fulfil the characteristics of phagolysosomes

The formation of particles with the above mentioned characteristics of phagolysosomes occurred only if bacteria were exposed to intact leucocytes No such particles were formed if bacteria were exposed to leucocyte post nuclear supernates containing lysosomes and soluble lysosomal enzyme (*Tagesson et al* to be published) The association phenomenon of bacteria and lysosomal constituents is thus not one of simple adsorption

In our study the sedimentation coefficient observed for phagolysosomes was lower than that of both the primary lysosome and that of the bacterium both being important constituents of the phagolysosomes Hence, additional lighter components have probably been added to the phagolysosome Membrane material stemming from the plasma membrane contributes to the membrane of the phagolysosome Such membrane material might be lighter than the lysosomal membrane either because of pre existing differences or as a consequence of de novo synthesis of lecithin rich material in the engulfment process (3) Alternatively, the content of the phagolysosome might have become lighter as a consequence of interiorization of lighter, extracellular material at the engulfment step or breakdown of macromolecular components leading to osmotic swelling

Quantitative evaluation of the data is difficult since discrimination between the differences between phagocytosing and non phagocytosing leucocytes both in their resistance to mechanical stress and in the fragility of their respective subcellular components deserves separate analytical experimentation However, one particular point may be stress

ed The significant amount of non sedimentable enzyme activity in the experimental situation is in sharp contrast to controls in which soluble enzyme constitutes a comparatively small amount of the enzyme activity These data would suggest that during phagocytosis, either lysosomal enzymes are released into the soluble fraction or lysosomal enzyme bearing particles are more readily disrupted by homogenization Variations in the fragility of lysosomal membranes have been observed in relation to the functional state of lysosomes (14) Lysosomes are considered especially fragile after the ingestion of exogenous material (2, 15) There are speculations that the increased fragility reflects altered membrane composition of such lysosomes which differs from that of primary lysosomes due to additional cell membrane components *Straus* (14) has even put forward the possibility that "membranes of 'old lysosomes', 'young lysosomes' and lysosomes soon after their fusion with phagosomes behave quite differently"

When the sedimentation pattern of the post nuclear supernate of phagocytosing plus non phagocytosing PMN cells (Fig 1) is compared with that of non phagocytosing PMN cells only (*Tagesson et al*, to be published), the enzyme content of the A region of the former (Fig 1C, $\rho = 1\ 25$) is relatively small This could be due to loss of particle bound activity to the sediment before zonal centrifugation as a consequence of homogenization with a loose fitting pestle (*Tagesson et al*, to be published)–the loss of soluble enzyme is probably less affected Alternatively, phagolysosomes could recruit further lysosomes or activation of hydrolytic processes in phagolysosomes could lead to release of enzyme activity from lysosomes not engaged in phagocytosis

Earlier experiments with a view to preparing phagolysosomes have employed artificial prey as emulsified paraffin oil (12, 13) which enabled ready separation of phagocytic vesicles by flotation *Werb* & *Cohn* (16) isolated macrophage phagolysosomes according to *Wetzel* & *Korn* (17) in a study of plasma membrane synthesis in macrophages following phagocytosis of polystyrene latex particles where the polystyrene containing phagolysosomes accumulated at a lower density Such studies have produced important insights into the functioning of phagocytic cells The question whether these models are truly representative of host parasite interaction remains, however, unanswered The lysosomal markers used in our experiments have not shown any qualitative differences between the enzyme contents of phagolysosomes containing bacteria and those containing paraffin oil or polystyrene It can be anticipated though, that different kinds of prey elicit different reactions in the phagocyte Phagocytosed *S typhi* do not elicit the same extent of metabolic reaction as do less virulent bacteria (9) Furthermore, morphological studies by *Armstrong* & *D Arcy Hart* (1) suggest that intracellular survival of *M tuberculosis* in cultured macrophages is associated with a tendency to nonfusion of lysosomal granules with the phagosome Further analysis of the phagocytic process will, therefore, have to take into account the events when microbes are in dynamic interaction with phagocytes i e when the properties of both parasite and host are operating Although more complex, this appears to be a less artificial image of the physiological case, and thus more relevant to an understanding of the process as it occurs in vivo

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PRODUCTION OF COLD AGGLUTININS IN RABBITS INDUCED BY *MYCOPLASMA PNEUMONIAE*, *LISTERIA MONOCYTOGENES* OR *STREPTOCOCCUS MG*

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Intravenous injections of *Mycoplasma pneumoniae* as well as of *Listeria monocytogenes* and *Streptococcus MG* stimulated the production of CA in rabbits Absorption experiments indicated that these microorganisms possess antigens related to the red cell I antigen most strongly represented in *M pneumoniae* It is suggested that the CA production during primary atypical pneuomonia caused by *M pneumoniae* may be triggered off by an I like antigen present in this microorganism

Patients who suffer from pneumonia caused by *Mycoplasma (M) pneumoniae* often demonstrate a significant level of cold agglutinins (CA) Titration for CA may be used as a diagnostic supplement to the determination of antibodies to *M pneumoniae* CA are generally harmless to the patient, but in a few cases they may give rise to haemolytic anaemia (15)

CA which develop during a *M pneumoniae* infection belong to the IgM class of immunoglobulins and have the specificity of anti I, i e they react with the I antigen site of erythrocytes present in the vast majority of the adult population (15, 17, 4, 19) These anti I CA were shown to be distinct from the antibodies to *M pneumoniae* that develop simultaneously in the patient (16, 4)

The aim of the present investigations was primarily to attempt to determine what antigenic stimulus leads to the production of anti I CA during a *M pneumoniae* infection A preliminary report has been published (14)

Received 22 ii 73 Accepted 16 iii 73

MATERIALS AND METHODS

Microorganisms

Mycoplasma (M) pneumoniae strain Mac was cultivated in Roux flasks each containing 70 ml of Hayflick's medium (9) to which was added 0 05 M N 2 hydroxyethyl piperazine-N 2-ethanesulphonic acid (HEPES) The mycoplasmas grown as a sheet on the inside of the flask were washed harvested and prepared as the antigen for the indirect haemagglutination (IHA) test (11) and used without sonication The preparation of live mycoplasmas is described under Experiments

Streptococcus MG (Strep MG) strain NCTC no 8037 was cultivated in Todd Hewitt broth heat killed at 65° C for 60 minutes and washed four times in saline It was stored at 4° C as a saline suspension with merthiolate 1 10 000 and used both for vaccine and as antigen for serological tests and absorptions

The strain 1309/58 of *Listeria (L) monocyto*

genes type 4b originated from human cerebrospinal fluid and was kindly supplied by the Department of Diagnostic Bacteriology at this Institute It was cultivated in Thiamine Polymyxin Tryptose broth (1), heat killed at 65° C for 60 minutes and washed twice in PBS It was resuspended in PBS and stored at −30° C after the addition of merthiolate 1 10 000

Reagents

Phosphate buffered 0 15 M NaCl pH 7 2 (PBS) was used for suspension of antigens and dilution of sera unless otherwise indicated

Medium 199 was prepared according to the method described by *Morgan et al* (20)

Rabbits

Young albino rabbits weighing 2 500 3 000 g, were bled four times at weekly intervals before the first dose of antigen was given At the end of the experiment in which four rabbits were injected with live *M pneumoniae*, the animals were examined post mortem together with the four controls The lungs were examined for gross changes*, and cultured for mycoplasmas and bacteria

Antibody Titrations

Titration for CA was performed as described previously (8) or, in some of the absorption experiments by the micromethod of *Fetzi & Monger* (6) Autologous or isologous rabbit erythrocytes were used after preparation as indicated below under Absorption and elution experiments In some experiments human OI and $OI_{(cord)}$ erythrocytes were used

Antibodies to *M pneumoniae* were estimated by the indirect immunofluorescence (IF) test (9 12) by the indirect haemagglutination (IHA) test (11) or by the metabolic inhibition (MI) test (22) For the IF test a sheep anti rabbit globulin conjugated with fluorescein isothiocyanate (Progressive Laboratories Baltimore U S A) was used The conjugate was diluted in PBS pH 8 containing 10 per cent heat inactivated horse serum

Antibodies to *Strep MG* were titrated either by tube agglutination (23 9) or by the IF test Before immunization the agglutinin titre was < 8 As antigen for the IF test a drop of diluted bacterial suspension containing 10^5 cells per ml placed on each microscope slide and fixed by air drying otherwise the test was carried out as described above

Antibodies to *L monocytogenes* were tested by tube agglutination and IF test as described for antibodies to *Strep MG* *Listeria* agglutinins were not detected prior to immunization

* Kindly performed by cand med vet *H J Skovgaard Jensen* Experimental Animal Laboratory Statens Seruminstitut

In order to evaluate the antibody production during immunization consecutive sera from one animal, including prevaccination sera were tested simultaneously in the same titration experiment

Absorption and Elution Experiments

Antigens For absorption of sera with *M pneumoniae*, an antigen prepared as for the IHA test (11) was used The suspension was centrifuged at 10 000 g for 10 minutes and the supernatant discarded The antigen was thoroughly suspended in serum diluted 1 2 in PBS The amount used for 0 5 ml serum represented about 10^{10} colour changing units (c c u) (22) of *M pneumoniae* as measured on the day it was harvested from the Roux flasks (11)

The *Strep MG* cells used for absorption were centrifuged at 1000 g for 10 minutes and one volume of the packed cells was suspended in two to five volumes of the 1 2 serum dilution

Heat killed *Listeria* organisms were treated in the same way, one volume of packed cells was suspended in three to six volumes of the serum diluted 1 2

It was sometimes necessary to recentrifuge the separated absorbed serum at a higher g to free it from residual bacteria or mycoplasmas

When autologous *rabbit red cells* were to be used for absorption the blood was drawn in anticoagulant-citrate dextrose (ACD) just before immunization of the animal was started The ACD blood was stored at 4° C after the addition of merthiolate 1 10 000 and penicillin 500 U/ml On the day of the CA absorption experiment an adequate amount of the red cells was washed three times in PBS, the final mixture contained equal parts of pac [illegible]

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rabbits The ACD blood was stored for up to a week but otherwise treated in the same way as the *autologous red cells*

Absorption procedures Before absorption the sera were heat inactivated at 56° C for 30 minutes and diluted 1 2 in PBS Equal parts of diluted serum were added to the tubes containing the particular packed antigenic cells They were completely suspended and the tubes were incubated in an ice bath with occasional shaking either for 3 hours or overnight The temperature of incubation was maintained while the suspensions were centrifuged mycoplasmas at 10 000 g bacteria at 1000 g and red cells at 500 g The absorbed supernatant serum was transferred to fresh antigen of the same batch for a second absorption and the procedure repeated

Elution procedures After the last cold separation red cells from the two consecutive CA absorptions were mixed suspended in about 10 times the

volume of PBS and incubated at 37° C for 30 minutes in order to wash out serum components trapped in the agglutinates The tubes were then transferred to an ice bath for 3 hours or overnight for reabsorption of the CA The red cells were spun down at 4° C and the supernatant discarded The thermal washing was repeated, followed by CA reabsorption, centrifugation and removal of the supernatant PBS was added corresponding to the original volume of diluted serum, and the CA were eluted at 37° C for 30 minutes and separated by centrifugation at that temperature

The same methods were applied in the attempts to elute CA adsorbed to *M pneumoniae*, *Strep MG* and *Listeria*

Experiments

Treatment of erythrocytes with live M pneumoniae *and subsequent intravenous injection* When the broth in 10 Roux flasks started to turn orange, empirically corresponding to about 10^6 c c u per ml of *M pneumoniae*, it was tested for possible contamination with bacteria or other species of *Mycoplasma*, and discarded The sheet of colonies on the glass was washed three times with medium 199 containing penicillin 1000 U/ml, and harvested with a 'rubber policeman' The colonies from each Roux flask were suspended in a tube containing 7 ml medium 199 Washed red cells from 0 5 ml blood drawn from one rabbit were mixed with the live mycoplasmas, and 0 3 ml of prevaccination serum from the same rabbit was added to the tube Simultaneously, red cells from 9 further rabbits were treated in the same way The suspensions were then checked for growth of *M pneumoniae* and absence of contaminating microorganisms The tubes were rotated in a 'roller drum' at 37° C overnight. Red cells from 10 other rabbits which served as controls were treated in the same manner with the omission of the mycoplasma culture

After incubation the red cell/mycoplasma suspensions were observed microscopically for haemadsorption (18) and peroxide production (13), the latter after adding a loopful of 0 1 per cent methylene blue to one drop of suspension Incubation with live *M pneumoniae* generally resulted in a brownish colour of the red cell suspension which contrasted with the red colour of untreated erythrocytes

The suspensions with and without mycoplasmas were then injected intravenously into the corresponding 10 rabbits plus 10 control rabbits The whole procedure was repeated, such that eight injections were given at intervals of 2-4 days between days 23 and 43 of the experiment In addition to the four weekly bleedings prior to the injections the rabbits were bled at regular intervals 6 times between days 35 and 57 (cf Fig 1) The blood specimens were allowed to coagulate and were incubated at 37° C for 30 min before the sera were separated by centrifugation at about 32° C All sera were stored at -30° C

D rect intra ...

rabbit, suspended in 7 ml of medium 199 plus 0 3 ml of autologous prevaccination serum The suspensions were given intravenously to four rabbits shortly after preparation Each of four control rabbits received 7 ml medium 199 with 0 3 ml autologous prevaccination serum

Heat killed *Strep MG* cells suspended in saline were given intravenously to three rabbits in doses of 0 5 ml, containing about 10^9 cells

Another three rabb ...

rigidly adhered to for the animals immunized with these microbial antigens

RESULTS

Immunization Experiments

The experiments were designed to test whether the adsorption of *M pneumoniae* to erythrocytes (18) would result in the exposure or liberation of the red cell I antigen or a related antigen, or whether the mycoplasmas in themselves appeared to possess this antigen Rabbits were used because they are able to produce CA (7) and because their red cells have a strongly reactive I antigen (25, 24)

The main results were that all 10 rabbits given autologous red cells that had been incubated with live *M pneumoniae* responded with a significant rise in titre of CA, as measured with autologous erythrocytes The average response was a 6-fold rise in titre when the mean titre before immunization was compared with the mean titre of all specimens drawn after the start of immunization The highest levels of CA were generally found in the first three specimens drawn during immunization, i e on days 35, 38, and 42 (Fig 1) The maximal response in one particular rabbit was measured as a 20 fold rise in titre In the control group there was a slight rise in titre in 7 animals and a fall in 3, with an average 1 3 fold rise (Table 1) A compar-

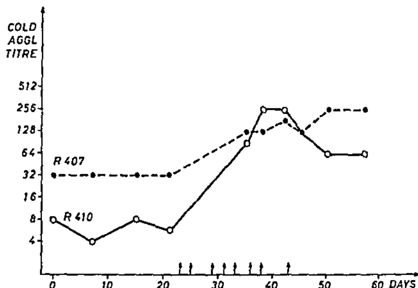


Fig 1 Cold agglutinin response in two rabbits † Injection of autologous red cells treated with *Mycoplasma pneumoniae*

able, but less pronounced, difference in CA titres between the two groups was obtained when CA were tested with isologous erythrocytes

In the control experiment (Table 2) the average rise in titre of CA in the four rabbits injected with mycoplasmas was again 6 fold when measured with autologous red cells Each rabbit gave at least a 4 fold response one of them showing maximally a 16 fold rise Tests with isologous erythrocytes also revealed a significant but less pronounced, rise There was no increase in CA titre in the control animals

Antibodies to *M pneumoniae* measured by IF rose in titre from about 10 to above 640 (maximum 5000) (Table 2) In the IHA test increases were from < 40 to between 640 and 10 000, and in the MI test from < 8 to between 640 and 40,000 None of the control animals which were injected with red cells or medium alone developed antibodies to *M pneumoniae*

The rabbits stimulated with live *M pneu*

TABLE 1 *Antibody Response in Rabbits Injected with Autologous Red Cells Treated with Live* Mycoplasma pneumoniae

Number of rabbits	Inoculum	Mean rise in titre of antibodies indicated: Cold agglutinins (autologous red cells)	Anti Mycoplasma pneumoniae IF	Anti Mycoplasma pneumoniae MI
10	*M pneum* + red cells in medium 199	6 fold	16 fold	> 200 fold
10	Red cells in medium 199	1 3 fold	no rise	no rise

IF = indirect immunofluorescence test
MI = metabolic inhibition test

TABLE 2 *Antibody Responses in Rabbits Injected Intravenously with Live* Mycoplasma pneumoniae

Number of rabbits	Inoculum	Mean rise in titre of antibodies indicated			
		Cold agglutinins	Anti M pneum IF	Anti Listeria IF	Anti Strep MG IF
4	*M pneum* in medium 199	6 fold	⋚ 64 fold	8 fold	⋚ 64 fold
4	Medium 199	No rise	No rise	NT	NT

IF = indirect immunofluorescence test
NT = not tested

moniae alone also developed significant amounts of antibodies to *L monocytogenes* and to *Strep MG*, simultaneous with the rise in titre of anti *M pneumoniae*, as indicated in Table 2

The rabbits were exsanguinated on day 57 On autopsy there were no macroscopic signs of pneumonia, and attempts to cultivate *M pneumoniae* from 4 representative sites in the lungs of each of the rabbits were unsuccessful

In an additional experiment three rabbits were repeatedly injected intravenously with a suspension of heat killed *L monocytogenes* type 4b, while another three rabbits were given intravenous injections of a suspension of heat killed *Strep MG*

In the rabbits that received *Listeria*, CA titres rose from the habitual low level about 32, to a maximum of 4 fold, 14 fold, and 20 fold, respectively (Table 3) There was no rise in titre of antibodies to *M pneumoniae* or to *Strep MG*, whereas *Listeria* agglutinins which could not be detected prior to the vaccinations, rose to high levels

In the three rabbits injected with *Strep MG* the CA titre rose 3 7 fold in the first (424) and 6 fold in the second rabbit (425) while there was no rise in the third animal (426) (Table 4) All rabbits formed agglutinins against *Strep MG* at a high titre The first rabbit responded with a 4 fold rise in titre of anti-*M pneumoniae* measured by IF, the third showed a 2 fold response (result of 6 titration experiments), while the second rabbit did not respond at all *M pneumoniae* antibodies were, however, not detected by the IHA or MI test In all three rabbits there was a significant rise in titre of *Listeria* antibodies measured by IF

TABLE 3 *Antibody Responses in 3 Rabbits Injected Intravenously with Heat killed* Listeria monocytogenes *Type 4b*

Rabbit number	Rise in titre of antibodies indicated		
	Cold agglutinins	Listeria agglutinins	Anti M pneum. (IF, IHA, MI) and anti Strep MG (IF)
421	4 fold	> 128 fold	No rise
422	14 fold	> 32 fold	No rise
423	20 fold	> 64 fold	No rise

IF = indirect immunofluorescence test
IHA indirect haemagglutination test
MI = metabolic inhibition test

TABLE 4 *Antibody Responses in 3 Rabbits Injected Intravenously with Heat killed* Streptococcus MG

Rabbit number	Rise in titre of antibodies indicated			
	Cold agglutinins	Strep MG *agglutinins*	Anti M pneumoniae IF	Anti Listeria IF
424	3 7 fold	> 64 fold	4 fold	16 fold
425	6 fold	> 128 fold	No rise	32 fold
426	No rise	> 64 fold	2 fold	8 fold

IF = indirect immunofluorescence test

Absorption Experiments

In order to elucidate the antigenic relationship between these microorganisms and rabbit red cell antigens, some absorption elution experiments were made

Table 5 gives the results of a typical experiment Three different rabbit sera were absorbed twice in the cold with isologous rabbit erythrocytes The first serum (444) was from a rabbit given live *M pneumoniae* intravenously, the second serum (424) was from a *Strep MG* immunized rabbit, and the third (421) from a *Listeria* immunized animal

In all three sera the CA were almost totally absorbed by red cells in the cold and quantitatively eluted from these at 37° C These absorptions did not affect the titre of antibodies directed against the microorganisms with which the animals had been immunized, nor did they affect the cross reacting microbial antibodies (Table 5) It should be noted that the relatively low anti *M pneumoniae* titre of 40 in the eluate from the red cells that absorbed serum 444 is negligible because of the unavoidable slight carry over during the washing procedure Furthermore it should be mentioned that the anti *M pneumoniae* titre of 20 of rabbit 421 was also found in sera prior to the vaccination

In the next series of experiments the obvious relationships between antigens responsible for the formation of microbial antibodies were investigated Sera from rabbits immunized with *M pneumoniae* were absorbed with either this organism, with *Listeria*, or with *Strep MG* The results of a typical experiment are shown in Table 6 In addition to

TABLE 5 *Absorption Elution of 3 Different Rabbit Antisera with Isologous Red Cells*

Rabbit no immunized with	Rabbit antiserum	Cold agglutinin titre	Anti M pneum IF titre	Anti Strep MG IF titre	Anti Listeria IF titre
444	Control	714	*5000*	640	160
Mycoplasma	Absorbed at 4° C	30	*5000*	640	*160*
pneumoniae	Eluted at 37° C	636	*40*	5	< 5
424	Control	84	320	*5000*	40
Streptococcus	Absorbed at 4° C	< 8	320	*2560*	40
MG	Eluted at 37° C	57	< 5	< *10*	< *5*
421	Control	76	20	< 10	*1280*
Listeria	Absorbed at 4° C	< 8	20	NT	*1280*
monocytogenes	Eluted at 37° C	118	< 5	NT	< *5*

IF = indirect immunofluorescence test
NT = not tested

TABLE 6 *Absorption Elution of a Rabbit Anti* Mycoplasma pneumoniae *Serum with Different Antigens*

Serum absorbed at +4° C with	Titre			
	Cold agglutinin	Anti M pneum IF	Anti Listeria IF	Anti Strep MG IF
Nil (control)	128	5 000	80	2 560
Erythrocytes (isologous)	< 4	10 000	80	1 280
Mycoplasma pneumoniae	8	*5*	≦ 5	5
Listeria monocytogenes	32	5 000	*20*	1,280
Streptococcus MG	48	5 000	80	*20*
37° C eluates from				
Erythrocytes (isologous)	128	20	< 5	10
Mycoplasma pneumoniae	≦ 4	20	< 5	20
Listeria monocytogenes	4	20	< 5	10
Streptococcus MG	< 4	20	< 5	< 5

IF – indirect immunofluorescence test

CA, this rabbit had developed antibodies to the three microorganisms mentioned In order to estimate the effect on CA absorptions were performed in the cold As expected each kind of microorganism absorbed anti bodies directed against itself, just as red cells absorbed the CA Furthermore, *M pneumoniae* removed all antibodies both to *Listeria* and to *Strep MG* as measured by IF, while *Listeria* and *Strep MG* were relatively in effective absorbants of cross reacting micro bial antibodies under the conditions of the experiment Unexpectedly CA were signifi cantly absorbed at 4° C by *M pneumoniae,* thus indicating its possession of an I like anti gen The CA were not eluted from the myco plasmas at 37° C Cold absorptions with *Listeria* caused a 4 fold decrease in CA titre, while *Strep MG* did not significantly absorb CA This type of absorption experiment gave similar results on several occasions

The possibility was considered that CA might be absorbed by *M pneumoniae* in the cold either as a non specific 'co-absorption' together with anti *M pneumoniae,* or due to trapping between the agglutinated myco plasma cells In order to investigate this eluates from red cells used for absorption of sera from rabbits immunized with *M pneumoniae* were subjected to absorption with the latter organism Prior to this absorption the eluates had a high titre of CA and a significantly reduced, low titre of antibodies to *M pneumoniae,* and no anti *Listeria* CA were removed from these eluates by absorp tion both with *M pneumoniae* and with *Listeria* in the cold

Similar tests were performed with eluates from red cells used for cold absorption of sera from rabbits immunized with *Listeria* The eluate from these cells contained CA a very low titre of *Listeria* antibodies, and no de tectable antibodies to *M pneumoniae* On absorption of this eluate with *Listeria* in the cold CA were significantly reduced, while cold absorption with *M pneumoniae* general ly resulted in about a 2 fold reduction in CA titre

Sera from two of the rabbits were assayed for anti I and anti i (17) The blood samples were drawn before and during a vigorous CA production while the animals were stimulated with autologous red cells treated with *M pneumoniae* The sera were absorbed once at 22° C and once at 37° C with human OI erythrocytes in order to remove heterophil antibodies The absorbed sera were then tested for CA both with human OI and with human $Oi_{(cord)}$ red cells The CA titres were in all cases lower with $Oi_{(cord)}$ red cells, indicating that CA in the rabbits were pri marily anti I (19)

Another observation was that 5 of the 10 rabbits which received their own red cells treated with *M pneumoniae* developed significant titres of warm haemagglutinins as demonstrated with autologous red cells at 37° C Using the same method, it was not possible to detect warm haemagglutinins in any of the 4 rabbits that received *M pneumoniae* directly intravenously, or in any of the 14 control rabbits given autologous red cells, or medium 199 alone

In addition, sera from two patients who suffered from CA positive primary atypical pneumonia were investigated in the same type of absorption experiment Serum (K 1699/72) from one patient had a high titre of antibodies to *M pneumoniae*, while such antibodies could hardly be detected in serum (K 3677/70) from the other patient Thermal absorption and elution of CA from these sera were performed with human group OI erythrocytes Absorption of the eluates with *M pneumoniae* in the cold resulted in an insignificant reduction (2 fold) in CA titre of the first specimen (K 1699/72) and a 4 fold reduction in the second (K 3677/70)

Finally CA were isolated from a serum* from a patient Da with the cold agglutinin syndrome The serum gave a negative test for antibodies to *M pneumoniae* A CA containing eluate prepared by human OI red cells was absorbed with rabbit red cells which caused a 16 fold reduction in CA titre when measured with human OI erythrocytes Ab[illegible]rption with *M pneumoniae* reduced [illegible] CA titre at least 4 fold while *Lister[illegible]a* [illegible] were unable to absorb these CA

DIS[illegible]SSION

The results show [illegible] intravenous injections of autologous red c[illegible]ll[illegible] treated with *M pneumoniae* as well as [illegible]njections of either *M pneumoniae* *L m[illegible]nocytogenes* or *Strep MG* alone are able to provoke the formation of CA in rabbits In addition *M pneumoniae* *Listeria* and, inconstantly *Strep MG* absorbed CA at 4° C, indicating that the three different kinds of microorganisms possess antigens related to the red cell I antigen This I like antigen was most strongly represented in *M pneumoniae*, less in *Listeria* and least in *Strep MG* The CA found in the rabbits were probably anti I, since *Wiener et al* has shown (25) that rabbit red cells have a very strong I antigen but lack the *i* antigen This was confirmed in a few experiments with human OI and $OI_{(cord)}$ red cells The CA formed in rabbits stimulated with the above microorganisms have been shown to be IgM immunoglobulins (2)

In somewhat similar investigations by *Feizi & Taylor Robinson* (4) the production of CA was elicited in 9 out of 28 rabbits The rabbits were injected with *human* erythrocytes that had been treated with *M pneumoniae* The authors did not succeed in raising CA in rabbits by injection of the animals own red cells treated with *M pneumoniae* or by injection of the organisms alone Their conclusion was that a reaction product of the human OI antigen and *M pneumoniae* might be responsible for the positive results

In the present investigation the injection of live *M pneumoniae* alone resulted in CA responses very similar to those elicited by injection of autologous red cells pretreated with live mycoplasmas This points to an effect of the microorganisms alone rather than one of a red cell mycoplasma interaction product It cannot be excluded that such an interaction might take place after intravenous injection of live *M pneumoniae* alone although the specific antibodies thereby elicited would tend to inhibit the adsorption of mycoplasmas to the red cells (18) However *L monocytogenes* and *Strep MG* do not adsorb to red cells which suggest that the CA triggering I like antigens are present on these microorganisms This suggestion is supported by the results of the absorption experiments

The microorganisms employed also possessed inter related antigens as expressed by the more or less cross reacting microbial antibodies which were elicited in the rabbits together with the CA The extent to which

* Kindly provided by professor *Morten Harboe* Oslo Norway

these antibodies cross reacted was not further explored During previous investigations of the antigenic relationship between *M pneumoniae* and *Strep MG* the author was unable to demonstrate antibodies to *Strep MG* in sera from rabbits immunized with killed *M pneumoniae* (10) As discussed in that paper, and as indicated by the present experiments, the antibodies to *Strep MG* are probably raised only by live *M pneumoniae*

The microbial antigens involved in this study were apparently not present on the rabbit erythrocytes which were unable to absorb the microbial antibodies The experiments confirmed the observations made by others (16 4) and mentioned in the introduction that the CA and the microbial antibodies are represented by different globulin molecules

Costea et al (2) performed experiments which were similar to some of the present work but which led to somewhat different results They stated for example that CA isolated by thermal elution from erythrocytes of rabbits injected intravenously with *M pneumoniae* also gave a positive complement fixation reaction with *M pneumoniae* antigen The same CA also agglutinated suspensions of *Strep MG* and *Listeria* The antibodies might have been trapped between agglutinated erythrocytes during the cold washing as the authors describe it and hence might have been present in the eluate

Recent investigations by the same group (3) showed that CA produced by rabbits challenged with *M pneumoniae Strep MG* or *L monocytogenes* were absorbed by lipopolysaccharide fractions of these microorganisms CA from patients with *M pneumoniae* infection were not inactivated by the intact microorganism but by a lipopolysaccharide fraction of it CA from patients with lymphoproliferative disorders were not absorbed by either

Various explanations of the antigenic stimulus that gives rise to CA production have been proposed in the course of time, including those based on the author's previous experiments (4 5 3 15) The present results support the idea that the development of CA during a primary atypical pneumonia caused by *M pneumoniae* may be triggered off by antigens possessed by this microorganisms

The I antigen has been found as an erythrocyte factor in several primates and lower species, and its distribution together with that of the i antigen cuts across taxonomic lines (25) The finding that I like antigens are also present in microorganisms is an analogue to bacterial antigens cross reacting with various human blood groups (21)

The development of warm haemagglutinins in some of the rabbits may be a parallel to autoimmune manifestations of *M pneumoniae* infection in man as for instance the development of antibodies to lung liver and brain (cited in 15)

The author is indebted to Mrs *Inge Dyre Zissack* and to Mrs *Antoinette Schneekloth* for skilled technical assistance

The statistical calculations were performed by *S Olesen Larsen* M A (Econ) Biostatistical Department Statens Seruminstitut

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A GENETIC STUDY OF INDUCIBLE ERYTHROMYCIN RESISTANCE IN *STAPHYLOCOCCUS AUREUS*

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The genes responsible for inducible erythromycin resistance in a set of DU 4916 strains of *Staphylococcus aureus* were eliminated spontaneously or after exposure to ethidium bromide or heat. In transductions they were transferred into wildtype (rec⁺) and recombinationless (rec⁻) strains at similar frequencies. If rec⁺ strains were used as recipients, the transfer frequencies of inducible erythromycin resistance would not be stimulated after increasing doses of ultraviolet irradiation of the phage lysate, whereas the transfer frequency of chromosomal cadmium resistance would be tenfold stimulated. These facts support the suggestion that the genes for inducible erythromycin resistance are carried on a plasmid. Treatments with acriflavine or ethidium biomide could not elucidate the connection with methicillin and inducible erythromycin resistance.

Two phenotypes of resistance to the macrolide antibiotics, inducible and constitutive, have been described in *Staphylococcus aureus* (11, 17, 38). The inducible one, which is isolated only from clinical specimens (38), confers sensitivity to oleandomycin and other macrolides in the absence of erythromycin. In the presence of erythromycin, however, the strains become resistant not only to the inducer but also to other members of the macrolide group (39) and to the lincosamides (6, 18) and the streptogramin B group (9). These drugs are all known to be inhibitors of the 50 S ribosomal subunit (34, 35, 38). *Weisblum et al.* (40) have suggested that the nature of inducible erythromycin resistance is a result of a production of altered ribosomes with a decreased ability to bind the drug to its 50 S subunit. The authors isolated mutants with constitutive resistance from an inducible strain and suggested that these mutants possessed a constitutively derepressed mutation of the inducible resistance gene(s). Constitutive resistance does not require erythromycin to express resistance to any of the macrolides (17, 38). A genetic determinant for constitutive erythromycin resistance localized on a penicillinase plasmid has been described (22, 25). There is also indirect evidence that the genetic determinant for inducible erythromycin resistance is localized on a plasmid since it can be eliminated by diethylsulphate (10) and can be transduced to a recombinationless (rec⁻) mutant of *Staphylococcus aureus* (27). Rec⁻ strains are unable to repair or to integrate DNA into its chromosome and only DNA which does not require integration is expressed, i.e. plasmid DNA (19).

In a previous report (13), a strain of *Staphylococcus aureus*, sensitive to erythromycin but resistant to methicillin was described. After treatment with acridines methicillin resistance was eliminated but inducible

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erythromycin resistance developed by an unknown mechanism. This paper reports on the genetic marker for inducible erythromycin resistance both with regard to its genetic character and to its connection with methicillin resistance.

MATERIAL AND METHODS

Strains of Staphylococcus aureus. The strains used are denoted as follows:

1. DU 4916 (pen⁺ met-r ero-s). This is a wild strain which produces penicillinase, is resistant to methicillin, but sensitive to erythromycin.
2. DU 4916 (pen⁺ met-s ero-r_i). This strain produces penicillinase, is sensitive to methicillin but inducibly resistant to erythromycin.
3. DU 4916 (pen⁻ met-s ero-r_i). This strain produces no penicillinase, is sensitive to methicillin but inducibly resistant to erythromycin. Strains 2 and 3 were obtained after treatment of strain nr. 1 with acridines, and the whole system has been described in detail earlier (13).
4. RN 8325 N. The bacterial cell of this strain is plasmid free, sensitive to most antibiotics and has been described elsewhere (23).
5. RN 1030 (rec⁻). This strain is a recombinationless mutant of strain 8325, i.e. plasmid bound genes can be transduced into the bacterial cell, whereas chromosomal genes cannot. It has been described by *Smith & Novick* (32). These latter two strains were kindly supplied by *Dr. Richard Novick*, The Public Health Research Institute of the City of New York.
6. AC 17855 (pen⁺ ero-r_c). This strain harbours an α plasmid responsible for penicillinase production and constitutive erythromycin resistance, and has chromosomal genes for resistance to arsenate and cadmium ions (15). The strain was kindly supplied by Dr. *Elizabeth Asheshov*, Public Health Institute, Colindale, London.

All phenotypic characteristics of these strains are listed in Table 1.

Sensitivity tests. The disc diffusion test as described by *Ericsson* (16) was used for antibiograms.

With a view to showing inducible erythromycin resistance, a disc diffusion test according to *Weisblum & Demohn* (39) was used. On a plate seeded with the appropriate test strain, three discs, impregnated with spiramycin, erythromycin or oleandomycin (10 μg per disc) were placed within about 1 cm of each other. After overnight incubation at 37° C, a decreased radius of the inhibition zone is seen on the side between the spiramycin and erythromycin discs and between the erythromycin and oleandomycin discs. This is illustrated in Fig. 1. Minimum inhibitory concentration (M.I.C.) for antibiotics was determined by a plate dilution test (5) as the lowest concentration of the drug which inhibits growth completely. A barely visible haze or a single colony is disregarded.

Assay of enzymes and toxins as coagulase, DNAase, haemolysins, lipase and enterotoxin B were performed as described elsewhere (12).

Detection of penicillinase production. The starch agar method described by *Dyke et al.* (14) was used.

Treatment with acriflavine was performed as described elsewhere (12). In attempts to induce erythromycin resistance, the nutrient broth (Difco, Detroit, Michigan, U.S.A.) with acriflavine was also inoculated with 0.1 μg erythromycin per ml. After incubation, the cultures were spread on nutrient agar plates (Difco) containing 10 μg erythromycin per ml.

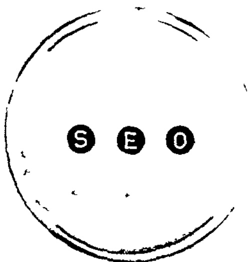
Treatment with ethidium bromide (BDH Chemical Ltd., Poole, England) was performed according to *Bouanchaud et al.* (8). Starting with an initial inoculum of 10^6 colony forming units per ml, cells were incubated during shaking for 24 hours at 37° C in Brain Heart Infusion broth (Difco) containing ethidium bromide. For strain DU 4916 pen⁺ met-s ero-r_i, 80 μg per ml was used and for strain DU 4916 pen⁻ met-s ero-r_i, 10 μg per ml. After incubation and suitable dilution the cultures were plated on nutrient agar (Difco) plates and replicated to drug agar plates (erythromycin 10 μg/ml agar, penicillin 10 μg/ml agar). After incubation overnight at 37° C, drug sensitive colonies were picked.

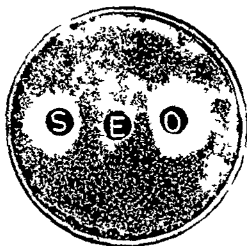
Loss of inducible erythromycin resistance after growth at 44° C. The procedure was performed as described by *Asheshov* (2). Replica plating on nutrient agar plates with (10μg/ml of erythromycin) and without drug was used to isolate sensitive colonies.

Phage typing was carried out by the method of *Blair & Williams* (7), using the standard set of typing phages and phage 88.

Propagation of phages for transductions. Typing phage 29 was used as the transducing vector in all experiments. It was propagated in the lytic cycle on the donor strains by the soft agar overlay method (33) and sterilized by membrane filtration (0.45 μm, Millipore Corp., Bedford, Mass.). Phage titres were determined in plaque forming units (p.f.u.) per ml and ranged between 5.0×10^9 to 1.0×10^9.

Transductions were performed according to the method of *Asheshov* (3). For selection, nutrient agar plates with 10 μg erythromycin per ml agar, 10 μg benzylpenicillin per ml or 7.5×10^{-5} M cadmium sulphate per ml were used. In transduction of inducible erythromycin resistance, subinhibitory concentration of the antibiotic (0.1 μg/ml) was added to the phage—recipient mixture during phenotypic expression (150 min). The





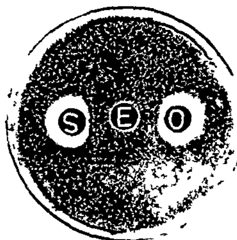


Fig 1 A disc test for wild strain DU 4916 pen met r ero s (left), mutant strains pen⁺ met s ero r_i (right) and pen met s ero-r_c (bottom) by which to show inducible erythromycin resistance
S Spiramycin 10 μg/disc
E Erythromycin 10μg/disc
O Oleandomycin 10μg/disc

selective plates were incubated at 37° C for 48 hours in transfer of ero r_i pen or Cd r The identity of around 20 transductants from each cross was confirmed by phage typing and antibiogram

The phage lysate diluted in nutrient broth, was also exposed to various doses of UV irradiation (a 6 watt UV lamp at a distance of 15 cm) The frequency of transduction was then plotted as a function of the dose of UV light given to the phage After irradiation samples were also titrated for survivors (p f u per ml) Transduction frequencies were calculated as the ratio of the number of transductants to the number of plaque forming units of the unirradiated lysate Controls including sterility test of the phage lysate and test for revertants in the absence of transducing phage, were performed with each experiment

RESULTS

It was noted earlier (13) that loss of the plasmid probably responsible for methicillin resistance and enterotoxin B production in strain DU 4916 resulted in a gain of inducible erythromycin resistance The resistance disappeared again if methicillin res stance were restituted by transduction With a view to obtaining additional insight into the genetic

TABLE 1 *Phenotypes of the strains used in the investigation*

Strain	Phage type	Antibiogram su	p	e	s	t	ch	a	m	c	Type of erythromycin resistance	M I C erythromycin µg/ml	M I C *methicillin* µg/ml	Type of *penicillinase* production
DU 4916														
pen met r ero-s	29/88 Gp III inhibitions	R	R	S	R	R	S	R	R	R	–	0.5	> 400	extrachromosomal
pen met s ero r_i	29/88 Gp III inhibitions	R	R	R	R	R	S	R	S	S	Inducible	1 × 10⁵	< 12.5	extrachromosomal
pen met s ero r_i	29/42E/47/53/54/75/88	R	S	R	R	R	S	S	S	S	Inducible	1 × 10⁵	< 12.5	–
RN 8325 N	29/52/52A/79/80/6/42E/ /47/53/54/75/77/42D/81/88	S	S	S	S	S	S	S	S	S	–	0.25	< 12.5	–
RN 1030 (rec)	29/6/47/53/54/75/77/88	S	S	S	S	S	S	S	S	S	–	0.5	< 12.5	–
AC 17855 pen ero r_c	29/52/80/77/88	R	R	R	S	S	S	R	S	S	Constitutive	2 × 10⁵	< 12.5	extrachromosomal

Abbreviations

pen⁺	penicillinase producing	rec	recombinationless strain of *S aureus*	Su	sulphonamide
pen	*penicillinase negative*			p	penicillin G
met r	methicillin resistant	M I C	Minimum inhibitory concentration	e	erythromycin
met s	methicillin sensitive			s	streptomycin
ero r_i	*inducibly erythromycin resistant*			t	tetracycline
ero s	erythromycin sensitive			ch	chloramphenicol
ero r_c	constitutively erythromycin resistant			a	ampicillin
				m	methicillin
				c	cephalothin

TABLE 2 *Curing of Inducible Erythromycin Resistance and Penicillinase Production in Two Strains of Staphylococcus aureus*

Strain	Curing method	Phenotypic characters lost	Number of* colonies
DU 4916	spontaneously	ero r_i	1
pen met s ero r_i		pen⁺	2
	ethidium	ero r_i	1
	bromide	pen	5
	heat	ero-r_i	1
		pen⁺	2
DU 4916	spontaneously	ero r_i	1
pen met s ero r_i	ethidium	ero r_i	1
	bromide		–
	heat		

Abbreviations as in Table 1
* With each curing method around 4000 colonies were scored

control of inducible erythromycin resistance, wild type strain DU 4916 and the mutants derived from it (Table 1) were used

Curing of inducible erythromycin resistance First we attempted to eliminate inducible erythromycin resistance by agents affecting the stability of plasmids A total of 3 sensitive mutants from strain DU 4916 pen⁺ met s ero-r_i and 2 *sensitive mutants of strain DU* 4916 pen met s ero-r_i were isolated spontaneously or after exposure to ethidium bromide or to heat (44° C) (Table 2 and

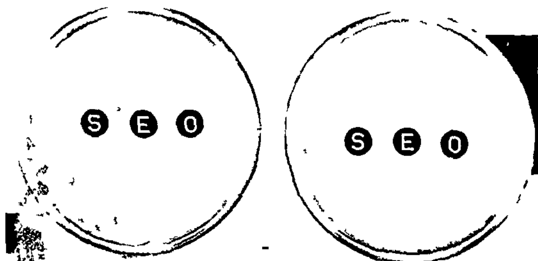


Fig 2 Curing of inducible erythromycin resistance in strain DU 4916 pen met s ero-r_i (left) after growth in ethidium bromide (right)
S Spiramycin 10 μg/disc
E Erythromycin 10 μg/disc
O Oleandomycin 10 μg/disc

TABLE 3 *Transduction of Inducible Erythromycin Resistance from Mutant Strain DU 4916 pen met s ero r, with phage 29*

Recipient strain	Frequency of transduction	Number of transductants	Frequency of transduction after UV irradiation of the lysate for 3 min (phage survival of 0 4)	Number of transductants
DU 4916 pen met r ero s	2 9 × 10 ⁶	2940	3 3 × 10 ⁶	3270
DU 4916 pen met s ero s (cured strain)	6 8 × 10 ⁶	6830	1 6 × 10 ⁶	6150
8325 N	4 7 × 10 ⁶	6136	4 8 × 10 ⁶	6938
RN 1030 (rec)	2 5 × 10 ⁶	3257	2 5 × 10 ⁶	3667

Fig 2) In parallel tests, a total of 9 penicillinase negative colonies were isolated from strain DU 4916 *pen⁺ met s ero-r₁ All ero-s segregants* from strain pen⁺ met s ero r₁ still *produced* penicillinase There was no change *in the* phage type pattern, antibiogram or in production of enzymes or toxins among the *ero s* segregants as compared to the parent *strains* The mutants were further used as recipients in transduction experiments

Transduction experiments With a view to investigating the nature of the genetic determinant for inducible erythromycin resistance transductions were performed with and without UV irradiation of the lysate In parallel it was attempted to transduce a penicillinase plasmid or chromosomal genes for cadmium *resistance into strain 8325 N or* RN 1030 (rec) to serve as controls for transfer of chromosomal and extrachromosomal genes The frequency of revertants in the absence of transducing phage was less than 10 ¹⁰ in all controls and a phage inoculum corresponding to a multiplicity around 1 0 was used in all experiments

a) *Transduction of ero r₁ and pen from strain DU 4916 pen⁺ met s ero r₁* Four recipient strains have been used DU 4916 pen met s ero-s DU 4916 pen met s ero s (the cured strain of pen met s ero-r₁ after growth in ethidium bromide), 8325 N and RN 1030 (rec) Inducible erythromycin resistant transductants were obtained in *all crosses* with frequencies of 2 9 × 10⁻⁶, 6 8 × 10⁻⁶ 4 7 × 10 ⁶ and 2 5 × 10 ⁶ respectively (Table 3) There was no co-transfer of penicillinase production It can also be seen from Table 3 that the rates of transduction were not significantly stimulated after UV irradiation of the lysate for 3 minutes which gave a phage survival of 0 4 Transduction rates of the ero r₁ or pen⁺ markers were then studied after application of increasing doses of UV light on the phage lysate the 8325 N strain being used as recipient Fig 3 shows that the *frequency* of transduction of ero-r₁ was not stimulated after high doses of UV light on

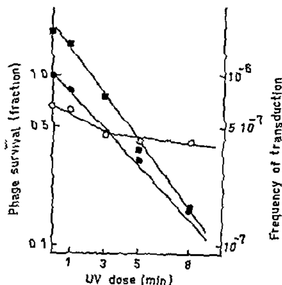


Fig 3 Transduction of inducible erythromycin resistance and penicillinase production from strain DU 4916 *pen met s ero r* into strain 8325 N with phage 29
● Phage survival
○ Transduction of ero-r
■ Transduction of pen

TABLE 4 *Transduction of Inducible Erythromycin Resistance from Mutant Strain DU 4916 pen met s ero r_1*

Recipient strain	Frequency of *transduction*	Number of *transductants*	Frequency of transduction after *UV irradiation of the lysate for* 3 min (phage survival of 0 4)	Number of *transductants*
DU 4916 pen met r ero s	$7\ 1 \times 10^{7}$	495	$5\ 9 \times 10^{7}$	412
DU 4916 pen met s ero s (cured strain)	$3\ 8 \times 10^{5}$	26300	$5\ 4 \times 10^{-6}$	37750
8325 N	*$5\ 3 \times 10$*	*370*	*$5\ 1 \times 10^{7}$*	*354*
RN 1030 (rec)	$2\ 6 \times 10^{6}$	1335	$3\ 9 \times 10^{6}$	1967

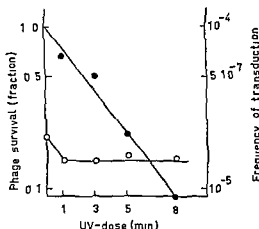


Fig 4 Transduction of inducible erythromycin resistance from strain DU 4916 pen met s ero r_1 into cured strain DU 4916 pen met s ero s with phage 29
- ● Phage survival
- ○ Transduction frequency of ero r_1

the lysate and that of penicillin resistance decreased exponentially The two markers were not co-transduced and a difference in dose response was visualized

b) *Transduction of ero r_1 from strain DU 4916 pen met s ero r_1* Also with this donor, inducibly erythromycin resistant transductants were obtained from the same four recipient strains with the frequencies of $7\ 1 \times 10^{-7}$, $3\ 8 \times 10^{5}$, $5\ 3 \times 10^{7}$ and $2\ 6 \times 10^{6}$, respectively (Table 4) No stimulation of the transduction rates was noticed after UV-irradiation of the lysate (0 4 phage survival) This is shown in Fig 4 where the rate is uninfluenced by high doses of UV-light with the cured strain DU 4916 pen met-s ero s as recipient

c) *Transduction of chromosomal genes for cadmium resistance from strain AC 17855* In order to check the rec strain efforts were made to transfer the chromosomal marker for cadmium resistance into it No transductants were found, not even after UV irradiation of the transducing lysate If the strain 8325 N were used as recipient, the marker could be transferred at a frequency of $4\ 0 \times 10^{7}$ and which would be ten fold stimulated after UV irradiation of the lysate (Fig 5)

d) *Transduction of pen⁺ ero r_c from strain*

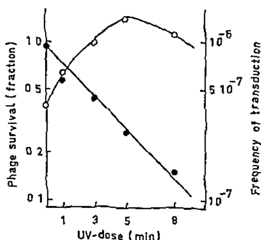
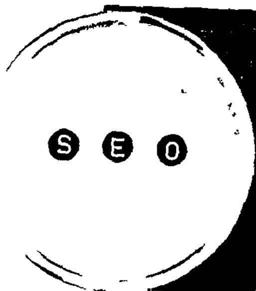


Fig 5 Transduction of chromosomal cadmium resistance from strain AC 17855 into strain 8325 N after increasing doses of UV light on phage 29
- ● Phage survival
- ○ Transduction of chromosomal cadmium resistance



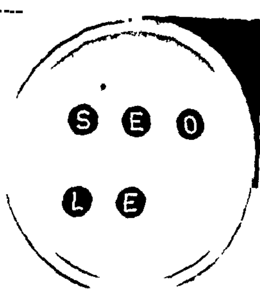


Fig 6 Development of constitutive erythromycin resistance from wild strain DU 4916 pen met r ero-s (left) after growth in 50 mcg acriflavine per ml (right)

S Spiramycin 10 µg/disc
E Erythromycin 10 µg/disc
O Oleandomycin 10 µg/disc
L Lincomycin 10 µg/disc

AC 17855 pen⁺ ero r_c to strain DU 4916 pen met s ero r_i To evaluate whether incompatibility (24) existed between the two types of erythromycin resistance in the staphylococcal cell, the penicillinase plasmid with constitutive erythromycin resistance was transferred to the pen met s ero r_i mutant of strain DU 4916 with a frequency of $1\ 9 \times 10^{-5}$

In M I C tests for erythromycin there was a slight difference between donor and recipient Because of the high values (Table 1) it was not possible to find additative M I C values of the transductants In the disc test the transductants showed no zones of inhibition around discs with spiramycin, erythromycin and oleandomycin like the donor After curing experiments at 44° C the penicillinase negative segregants showed inducible erythromycin resistance

Studies of the Possible Connection of Inducible Erythromycin and Methicillin Resistance in the DU 4916 System

a) *Transduction of ero r_i from wild type strain DU 4916 pen⁺ met r ero s* In attempts to reveal the possibly blocked marker for ero r_i phage 29 was propagated in the wild type strain and strains pen met s ero s and 8325 N were used as recipients in transductions Erythromycin resistant transductants were obtained with frequencies of $1\ 4 \times 10$ and $6\ 7 \times 10^{-8}$, respectively, with M I C ranging between 20–40 µg/ml They did not show the inducible phenotype There was no change among the transductants in phage typing or in antibiograms as compared to the recipients

b) *Treatment of wild type strain DU 4916 pen⁺ met r ero s with acriflavine or ethidium bromide* Since the wild strain developed ero r_i after curing of met r (13) it was of interest to investigate whether mutagenesis to ero r_i might be connected with loss of met r To induce erythromycin resistance in the wild strain (its M I C being 0 5 µg/ml) subinhibitory concentration of the drug (0 1 µg/ml) was added to the broth with acriflavine or ethidiumbromide Only colonies with low constitutive erythromycin resistance (M I C of 20µg/ml) were found at the low frequencies of $3\ 5 \times 10^{-8}$ and $0\ 6 \times 10^{-8}$ re-

spectively, on erythromycin agar plates (10 μg/ml) (Fig 6) Sensitivity to methicillin was also noticed according to the disc tes among these colonies (12/50 and 4/21, re spectively) Among the controls free from acriflavine or ethidiumbromide low consti tutive erythromycin resistant colonies were found at the frequencies of 0.3×10^{9} and 0.5×10^{9} respectively all of them still being methicillin resistant

DISCUSSION

The mode of action in curing *Staphylococcus aureus* from plasmids is similar for ethidium bromide and acridines i e intercalation be tween base pairs in the DNA helix (21, 29 31 36) This may form the basis for the in hibition of plasmid replication Growth at elevated temperatures causes loss of plasmids probably as a consequence of breakage from its maintenance site (2 24) In our experi ments (Fig 2), growth in ethidium bromide or at 44° C caused loss of inducible erythro-mycin resistance and penicillinase production which is in accordance with the results ob tained by other investigators (10 22) As the ero r_1 and pen$^+$ markers were lost separately and independently of each other, it can be concluded that they are on separate linkage groups It might be of interest that the strain pen$^+$ met s ero r_1 needs higher concentration of ethidium bromide than the pen met s ero r_1 strain This is in agreement with the report by *Johnston & Dyke* (20) who found a marker for resistance to ethidium bromide present on a penicillinase plasmid in a strain of *Staphylococcus aureus*

The inducible nature of ero r_1 and its transducibility by phage was first described by *Pattee and co workers* (28 38) Our transduction experiments confirm the sugges tion that the ero r_1 marker in the 4916 system is bound to a plasmid The high trans duction frequencies were not stimulated after UV irradiation of the lysate, using different recipients including the rec strain (Tables 3 & 4) Any genetic determinant that shows similar rates of transfer to rec$^+$ and rec reci pients is probably plasmid linked (19 26) It is noted that the cured recipient strain pen met s ero-s seems to have a memory' for ero r_1 since the marker is transferred at a ten fold higher frequency to this than to other recip ents Using the strain 8325 N or the cured strain DU 4916 pen met-s ero s as recipients the rates of transfer for ero r_1 were not stimulated after increasing doses of UV light on the lysate and that for penicillinase production decreased exponentially (Figs 3 & 4) The different behaviour suggest that the genes controlling ero r_1 are less sensitive to UV light than those controlling pen$^+$ The behaviour may also be due to the difference in size of the two plasmids to the base con tent of the two DNAs or to the difference in availability of the repair mechanism in the host (37 41) Furthermore it was not possible to transfer chromosomal genes for Cd r into the rec strain So far however it has not been possible to identify any plasmid DNA in CsCl/ethidium bromide gradients from a rec strain carrying the locus for inducible erythromycin resistance (27) This could be confirmed by using the technique with a transductant from the cross RN 1030 (rec) x Ø 29/DU 4916 pen$^+$ met-s ero r_1 with selec tion for ero r_1 The donor strain showed cir cular closed plasmid DNA as it also harbors a penicillinase plasmid (13), but neither the recipient nor the transductant showed this DNA peak (*Martin Lindberg* data not pub lished) However our data suggest that the genetic determinant for inducible erythro-mycin resistance is bound to a plasmid (1 3 4)

By transducing plasmid located constitutive erythromycin resistance (ero-r_c) into a reci pient with ero-r_1 the constitutive phenotype was found to be dominant The transductants also showed res stance to spiramycin and oleandomycin in the absence of erythromycin This is not the case among the penicillinase plasmids where a diploid cell with inducible and constitutive penicillinase production still produces the enzyme inducibly (24, 30) Since ero-r_1 reappeared in the transductants

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ACUTE OTITIS MEDIA

Assay of complement-fixing antibody against Haemophilus influenzae *as a diagnostic tool in acute otitis media*

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A complement fixation (CF) test employing a mixture of whole bacteria from twenty non capsulated *H influenzae* strains as antigen was used for studying antibodies against *H influenzae* in a group of patients with acute otitis media and in a group of children without signs of respiratory tract infection (RTI) In the group of 192 children (0 16 years of age) without RTI, CF titres were found in more than half the number of children under one month of age while such titres were not demonstrated in children aged 1 month—1 year Only a few children aged 1 2 years showed a CF titre, but in the subsequent age groups CF titres were found to be present at increasing frequency, up to about 80 per cent Sera from 141 patients with acute otitis media (96 children and 45 adults) were studied by means of samples generally obtained initially and after 10 days of penicillin therapy The analyses showed that the initial titres of patients in Group I (*H influenzae* not isolated) were similar to those of patients in Group II (*H influenzae* isolated initially and 10 days later) The same average titres were found in Group I in samples taken after 10 days while increased titres were registered for more than half the number of patients in Group II The titres of children and adults with otitis media were similar Patients whose recovery was delayed or poor showed titre increases less frequently than those who recovered within 10 days The results of the present study using a CF test employing whole non capsulated bacteria indicate the suitability of this test as a diagnostic aid in respiratory tract infection where non capsulated *H influenzae* is suspected to be the infecting agent

As previously reported, a group of patients with acute otitis media was studied with regard to aetiological and therapeutic aspects (7, 11) In accordance with other reports published in recent years (for ref, see 11), the bacteriological study showed that *D pneumoniae* and *H influenzae* were the pathogens most frequently isolated The results indicated that nasopharyngeal samples are of diagnostic value

Serological tests as a diagnostic aid in the diagnosis of *H influenzae* infections and in determining the prevalence of *H influenzae* antibodies at various ages have so far been used only to a limited extent For studies of antibodies against the capsular antigens of interest in e g *H influenzae* type b infections the indirect haemagglutination method has been applied in recent studies (1, 2, 10, 14 15, 17, 19) It has been reported in population studies that anti b antibodies are very frequently found In patients with *H influenzae* type b infections and in individuals immunized with purified substance b, an increase in titres against capsular substance b was found (2, 17)

For analyses of the antibody response to non capsulated *H influenzae* bacteria, more

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prevalent than capsulated strains at respiratory tract infections like sinusitis and acute otitis there exist no generally accepted routine methods Direct agglutination has not been commonly used due to the frequent occurrence of spontaneous agglutination and to the presence of a number of antigen variants in non capsulated *H influenzae* strains (16, 20, 21) *Tunevall* (3, 18) used a complement fixation test, employing a sodium carbonate extract from a non capsulated strain of *H influenzae* as antigen In a control group CF-fixing antibodies were demonstrated in the majority of healthy adults Such antibodies were also demonstrated in cord blood and in blood from children under the age of 3 months In the age group 3 months to 3 years CF fixing antibodies were demonstrated only occasionally in contrast to the age group 4 7 years in which such antibodies were present in about 80 per cent of the cases Children with acute otitis media from whom *H influenzae* was isolated had, on the average lower initial titres than healthy controls of the same age Some of these children over 3 years of age displayed increasing titres in the course of the infection (3 18) *Glynn* (9) using an indirect haemagglutination test with the same type of antigen preparation as that used by *Tunevall*, registered high titres in sera from patients with chronic bronchitis whereas such titres were uncommon in sera from patients with bronchial asthma and in healthy controls

Serological methods by which to measure the bactericidal activity of sera against *H influenzae* have also been used (1 2 8 14 17) It has been assumed that antibodies against the capsular substance show such activity (2 17 20) However measurement of the bactericidal activity of sera has not hitherto been used as a diagnostic aid

In a previous study one of the authors of the present report tried to evaluate the diagnostic usefulness of the complement fixation method employing whole *H influenzae* bacteria as antigen (6) It was found that superficial cell wall antigens—O antigens—of *H influenzae* are good immunogens capable of inducing demonstrable antibody formation in rabbits within a week after the first antigenic stimulation as demonstrated by direct agglutination, complement fixation, or double diffusion in agar gel The complement fixation test was found to be less specific than the direct agglutination tests, as illustrated by the results obtained when two strains with partly different O antigens were employed

The aim of the present study was to evaluate the diagnostic usefulness of the complement fixation test employing the type of antigen previously described (6) Rising titres in the course of, e g , an acute otitis media infection would support a presumptive *H influenzae* diagnosis based on isolations from nasopharyngeal samples In addition, information might be obtained concerning the humoral immunodefence against *H influenzae*

MATERIAL AND METHODS

Children Showing no Signs of Respiratory Tract Infection (RTI)

Sera from 192 children at ages varying from less than one month to 16 years were included in the study for comparison These children had been admitted to the Department of Pediatrics Goteborg for e g surgical treatment and controls of various kinds They had no recent history of RTI and were without clinical signs of respiratory tract infection

Patients with Acute Otitis Media

Serum samples were obtained from 141 of the 237 patients with acute otitis media previously reported on (11) The study included 96 children (3 16 years of age) and 45 adults

The patients were treated with either of two penicillin preparations phenoxymethylpenicillin and α azidobenzylpenicillin for 10 days (11) On the basis of the therapeutic results the patients were divided into the following groups

1 Patients who recovered within 10 days
2 Patients not considered recovered after 10 days (delayed recovery), neither anatomically nor functionally but in whom continued antibiotic therapy was not considered indicated
3 Patients who had not recovered after 10 days (treatment failures) and in whom continued antibiotic therapy (chosen according to the sen

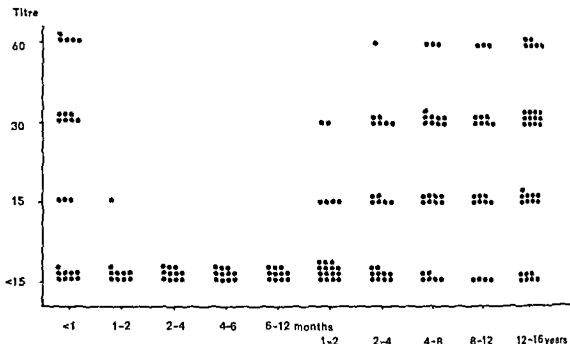


Fig 1 CF titres in sera from children of various ages without signs of RTI

sitivity test of the infecting strain) was considered indicated

The patients in the two latter groups were examined not only on the 10th day but also on the 20th

The blood samples were generally taken at the first examination and after 10 days In some cases samples were only obtained at the first or the second exam nation The sera were kept at −25° C until the tests were performed Samples for bacterial cultivation were obtained from the nasopharynx of all patients at the first examination and after 10 days of pen cillin therapy The bacterial diagnostic criteria have been described earlier (7)

Serological Tests

Complement fixation test (CF) The methodological detai s of the mode of analysis have been described in a previous report (6) For the present study a stock cell suspens on was prepared from equal parts of bacteria of twenty non capsulated strains (a series isolated from the patients comprised in the present st i ly in order to take into consideration possible O n gen variants among the infecting strains The teria had been cultivated for 6 h on AFH ag and were dispensed in buffered saline (pH 4 containing mert hiolate 1 10000 to OD at 1 in a Beckman C colorimeter The stock ce pension was stored at 4° C and before use di 1 to OD 0 5 The antigenic stability of the stock cell suspension was found to have a duration of at least one year

The CF test was performed with 0 1 ml of serum and the final serum dilution in the first tube was 1 15, further dilutions being performed in twofold steps The titres are reported as the dilution in the last tube with 50 per cent or less haemolys s (in the figs given as the reciprocals) All serum samples from one and the same pat ent were tested on the same occas on and positive and negative controls were included each time

Indirect haemagglutination test (IHA) Th s method was used in two variants I and II For the IHA variant I untreated sheep erythrocytes were used and for IHA variant II sheep erythrocytes pretreated with tannic acid according to the method of *Boyden* (4) For IHA I the erythrocytes were sensitized with capsular substance b for 2 h at 37° C the test be ng performed in the way described earlier (6) For the IHA II variant the sheep erythrocytes treated with tann c acid were sensitized for 30 min at 37° C with a somatic antigen mixture obtained by *extraction* of non capsulated *H influenzae* dis ntegrated in an X press (Biotec AB Stockholm) In other respects variant II was similar to var ant I

The test sera were analysed in series of twofold dilutions beginn ng with ¼ As controls for the IHA variant I, two rabbit anti *H influen ae* type b immune sera were employed (titres 1 16 400 and 1 524 300 respectively) As controls for the IHA variant II, two rabbit immune sera against

TABLE 1 *Patient Groups at the CF Tests The Groups are Based on the Results of Cultivation of H influenzae in the Nasopharyngeal Samples*

Group	Growth of *H influenzae*		Number of patients according to serum sampling			
	Sample I	Sample II	Total	I + II	I	II
I	—	—	68	41	22	5
II	+	+	50	25	17	8
III	+	—	18	16	2	0
IV	—	+	5	5	0	0

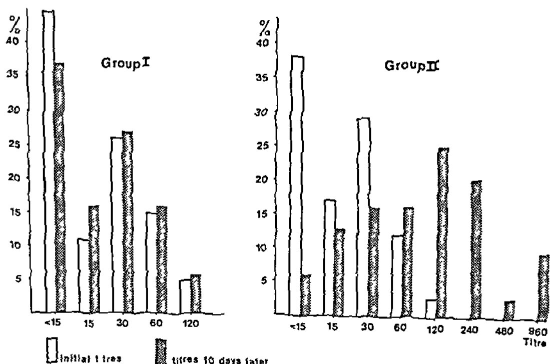


Fig 2a CF titres in sera from patients from whom *H influenzae* was not isolated (Group I)
Fig 2b CF titres in sera from patients from whom *H influenzae* was isolated initially and after 10 days (Group II)

somatic antigens were used (titres 1 16 400 and 1 131 100 respectively)

RESULTS

Children Showing no Signs of RTI

In order to obtain a general view of the prevalence of antibodies against non capsulated *H influenzae* in children at various ages sera from 192 children (newborn to 16 years of age) were analysed by means of the CF test

The material, grouped according to age and the titres found, is illustrated in Fig 1 It can be seen that titres were demonstrated in more than half the number of children The titres did not exceed 1 60 in any child However, the distribution of titres differed over the different age groups In the group of children less than *one month old*, titres

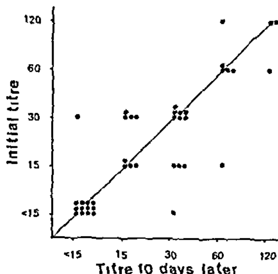


Fig 3 Comparison between the initial CF titres and the titres obtained after 10 days in sera from patients in Group I

ranging from 1 15 to 1 60 were seen in more than half the number of children, while only 1 out of 10 children in the age group 1 2 months showed a titre (1 15) In the age group 2 12 months none of the 33 children had a demonstrable CF titre (level <1 15) and only a few children in the group 1-2 years showed a titre (1 15 1 30) However, in the group 2-4 years and the group 4 16 years, CF titres ranging from 1 15 to 1 60 were found in about 50 per cent and 80 per cent of the cases, respectively

Patients with Otitis Media

The patients were divided into four different groups according by as *H influenzae* was demonstrated in the nasopharyngeal sample at the first and/or second examination or it was not demonstrated at all The groups and the number of patients in each group are given in Table 1 It should be mentioned that *D pneumoniae* and *Str pyogenes* were also found in a few patients from whom *H influenzae* was isolated, as will be reported later

Group I (*H influenzae* not isolated) consisted of 68 patients Matched serum samples were obtained from 41 of these, from 22 a sample was obtained only at the first examination, and from 5 only at the second Fig 2a summarizes the CF results obtained in Group I On admission nearly half the number of patients had no demonstrable titre (<1 15), the highest titre seen being 1 120 noted in only a very few patients In samples taken 10 days later the frequency of no de

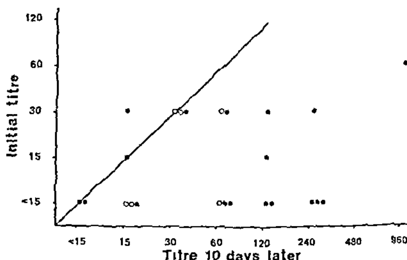


Fig 4 Comparison between the initial CF titres and the titres obtained after 10 days in sera from patients in Group II Open symbols indicate titres in sera from patients who did not recover within 10 days

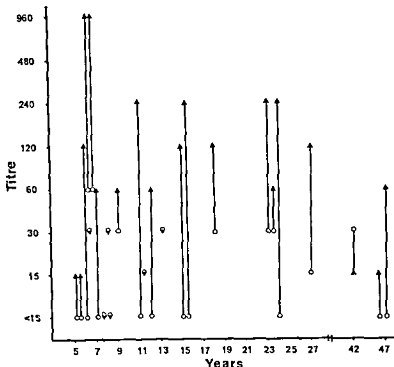


Fig 5 Age distribution of patients in Group II (*H influenzae* isolated initially and after 10 days) and their initial and subsequent CF titres ○ Initial titre, ▲ titre 10 days later, ♀ same titre initially and 10 days later

monstrable titre and that of demonstrable titre was of the same order as in the set of samples taken initially As can be seen from Fig 3, giving the results for the 41 patients from whom matched sera were obtained, any clear-cut tendency towards higher titres was not demonstrable

In Group I the ratio of children to adults was 42 26 It may be noted that 3 patients with an initial titre of 1 120 and 6 out of 8 patients with a titre of 1 60 were children while the frequency of absence of demonstrable titres (<1 15) was found to be about the same in children and adults

Group II (*H influenzae* isolated at the first as well as at the second examination) included 50 patients Matched serum samples were obtained from 25 of these, from 17 a sample was obtained only at the first examination and from 8 only at the second (Table 1) As can be seen from Fig 2b, the initial titres in the patients in Group II showed a distribution very similar to that in patients in Group I (Fig 2a) However, in the serum samples taken 10 days later there is a difference between the two groups, GroupII showing a marked shift towards higher titres Few patients had no demonstrable titre, while a titre as high as 1 960 was seen in about one tenth of the patients This shift is also obvious in Fig 4, where the titres in the 25 individuals from whom matched serum samples were obtained are illustrated It applies to about half the number of patients that a titre increase, fourfold or higher, was registered, five of these patients showing as much as a 16 32 fold titre increase It should be noted that in 5 (four children and one adult) out of 12 patients with no titre increase (twofold or lower) recovery was delayed or failure to respond to treatment was observed However, one child showed an increase in titre

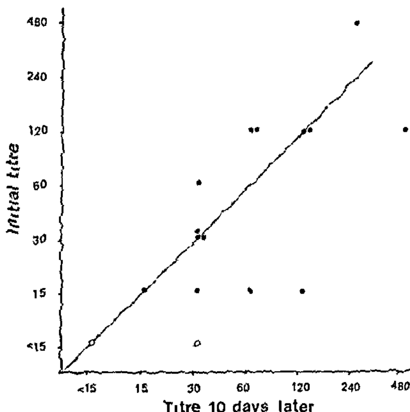


Fig 6 Comparison between the initial CF titres and the titres obtained after 10 days in sera from patients in Group III (*H influenzae* isolated initially but not after 10 days) Open symbols indicate titres in sera from patients who did not recover within 10 days

from <1 15 to 1 60 even through it failed to respond to treatment

It may be added that *D pneumoniae* was isolated in addition to *H influenzae* in the initial samples in three cases where matched serum samples had been obtained One of these patients showed a CF titre increase against *H influenzae* while the titre did not change in the remaining two (recovery was delayed in one of the latter)

In total Group II included 36 children and 14 adults The age distribution of the patients in Group II together with their initial and subsequent CF titres is illustrated in Fig 5 Low initial titres were found irrelevant to the age of the patient and the increase in titres was also unrelated to age

Group III (*H influenzae* found in the initial sample but not in the second) comprised 18 patients 16 from whom matched serum samples were obtained and 2 from whom only an initial sample was available The CF titres of the two latter patients were 1 60 and 1 120, respectively As appears from Fig 6 the initial titres of seven of the patients from whom two serum samples were obtained were 1 60 or higher A fourfold or greater increase in titre occurred in four cases It may be added that two children in Group III failed to respond to the treatment and that neither of them showed an initial titre (<1 15) one of them showing a titre of 1 30 in the second sample

From two patients in Group III *D pneumoniae* was isolated and from one patient in the same group *Str pyogenes* was isolated in addition to *H influenzae* in the initial samples The second samples showed no pathogens None of these patients demonstrated a CF titre increase against *H influenzae* Group II included 17 children and 1 adult

Group IV (*H influenzae* not found initial

ly but at the second examination) comprised only five patients Two of these had titres of 1 30 and 1 60 in both the first and second samples The remaining three patients had no initial titres (<1 15) One of these showed a titre of 1 30 in the second sample, while the other two remained without demonstrable titres

The results of the IHA tests showed that the IHA-I test was negative (titre <1 8) in about 90 per cent of all patients with otitis media The observed titres ranged from 1 8-1 32, but no titre increase (at least a fourfold difference in titres) was registered in their second samples An IHA-I titre was demonstrated in only 2 out of 7 patients from whom *H influenzae* type b was isolated, in one of these the change in titre ranged from <1 8 to 1 16 The IHA-II test was negative in more than half the number of patients, the observed titres ranging from 1 8-1 128 Only 4 patients from whom *H influenzae* was isolated initially and after 10 days showed a fourfold increase in titre It may be mentioned that these patients also displayed an increase in CF titre (4 32 fold)

DISCUSSSION

The indirect haemagglutination method (IHA) by which to measure anticapsular antibodies, has been found to be useful in serological studies of infections caused by capsulated *H influenzae* (1, 10, 15, 19) However, most *H influenzae* infections in the respiratory tract are caused by non capsulated bacteria and serological tests have only rarely been used in studies of such infections (3, 9, 18)

Earlier studies by one of the authors have shown that the CF test could be used for measuring antibodies against *H influenzae* O antigens (6) These studies further revealed that *H influenzae* O antigens were not fixed to untreated sheep erythrocytes This is in contrast to what has been found for e g *E coli*, where the O antigen is easily fixed to untreated sheep erythrocytes (12)

The direct agglutination method is not practicable in serological studies of the antibody response to non capsulated *H influenzae* due to *inter alia* the common occurrence of spontaneous agglutination For this reason the CF test was chosen for the present investigation, in order to determine the value of this method as a diagnostic aid in the study of infections possibly caused by non capsulated *H influenzae* The test was performed with whole bacteria as antigen and, in view of the variability of the O antigen of *H influenzae* strains, the antigen for the CF test was produced so as to contain twenty non capsulated *H influenzae* strains, isolated from patients examined in the present study

Tunevall (3, 18) applied a CF test, using an intracellular extract as antigen, to sera from children with acute otitis media and to healthy individuals at various ages In sera from children with acute otitis media, the increases in titre were found to be lower than those recorded in a similar series of patients tested by means of the CF technique used in the present study This difference might be explained by the fact that different types of antigen have been used *Tunevall* studied the antibody response to intracellular antigens, while the response to superficial cell wall antigen(s) was registered in the present study The latter antigens are efficient as immunogens, possibly due to e g their localization on the surface of the bacteria It may be mentioned that if the authors employed the IHA-II method, where intracellular antigens were used, it was found that more than half the number of patients with otitis media had no detectable titres, and also that a titre increase was registered only rarely The titres observed were quite low (≤1 28) as compared to those of the hyperimmunized rabbits (≥1 16,400) and to the titres found by *Glynn* (9) in sera from patients with chronic bronchitis (1 8,000 1 16,000) An intense artifical immunization or a severe protracted infection with *H influenzae* may be necessary for the development of high titres against *H influenzae* intracellular antigens

As a background study, the CF test was carried out on sera from a group of children

without signs of RTI, their ages ranging from newborn to 16 years The results showed that more than half the number of children under one month of age (mostly newborn) had demonstrable CF titres These titres were probably due to the presence of antibodies of the IgG type, transferred from their mothers In the age group 1-2 years, demonstrable titres were found in only a few children, but in the subsequent age groups, *H influenzae* antibodies were demonstrated at an increasing frequency, up to about 80 per cent The markedly increasing frequency of antibodies against *H influenzae* in the age groups 1-16 years indicates a phase of active immune response to *H influenzae* infection(s) in this period of life

The demonstrated changes of the CF titres against the O antigen during childhood are similar to the changes observed by *Tunevall* (18) He found CF antibodies against intracellular antigens of *H influenzae* in most samples of cord blood and in some of the children under 3 months of age As a rule such antibodies were lacking in children at ages from 3 months to 3 years In children aged 4 7 years CF antibodies were found in about 80 per cent of the cases the same frequency as that demonstrated in the present investigation

In this study titres not exceeding 1 30 were demonstrable in the majority of children without RTI, aged 2 16 years A titre of 1 60 was demonstrated in only 10 per cent of the cases Therefore, titres of 1 120 or higher seem to be above normal and may be regarded as signs of a possible *H influenzae* infection More conclusive evidence however, should be the demonstration of a definite titre increase

The titres of patients in Group I (*H influenzae* not isolated) from whom matched serum samples were obtained mostly children showed titres at about the same level as that in children without RTI However three children in Group I had an initial titre of 1 120 This observation could be an indication of a recent *H influenzae* infection or possibly a current infection with *H influenzae*, although the bacteria could have escaped detection

The initial titres of patients in Group II (*H influenzae* isolated initially and 10 days later) from whom matched serum samples were obtained, showed average titres that were slightly lower than those in Group I However, there was a marked difference between Groups I and II as regards the titres in the second set of samples In Group I about half the number still had no demonstrable titres, or titres of 1 15, while about half the number in Group II had titres of 1 120 or higher (Fig 2a and 2b), probably indicating a current *H influenzae* infection It should be mentioned that a presumptive diagnosis had earlier been established in patients in Group II by means of *H influenzae* positive nasopharyngeal samples

In comparison with the patients in Group II, those in Group III (*H influenzae* isolated only initially) showed, on the average, higher initial titres but less frequently a titre increase It may be of interest to note that the initial titres in patients with otitis media classified into Groups II III and IV, from whom matched samples were obtained were on the average, lower than those of patients with acute sinusitis from whom *H influenzae* had been isolated However in cases in which *H influenzae* had not been isolated the titre levels were similar in patients with otitis media and in patients with sinusitis (13)

The present results may be compared with those obtained by *Tunevall* (3 18) who employed a CF test using intracellular antigens for studies of children with acute otitis media He seldom found a CF titre in children under 4 years of age whether or not they were suffering from *H influenzae* infection From the age of 4 years, detectable immunization was frequent In some patients over 2 years of age titre increases were demonstrated during and after the otitis media infection The findings by *Tunevall* are similar to the results obtained in the present study where, however, otitis media patients under 3 years are not included

It is possible to assume that an initial high

CF titre or a CF titre increase to such a level is a sign of an effective immune defense against *H influenzae* This assumption is supported by the finding that, within Group II, there was a clear difference between the frequency in titre increases in patients who recovered within 10 days (12 out of 19) and patients whose recovery was delayed or poor (1 out of 6) Furthermore, the two patients in Group III who failed to respond to treatment lacked an initial titre, one of them revealing only a moderate titre in the second sample

In studies of the bactericidal activity of sera against *H influenzae*, *Fothergill* & *Wright* (8) showed that such activity against a strain isolated from a case of meningitis was generally found in infants under 2 months of age, in children over 3 years of age and in adults, while such activity was absent in children aged from 2 months up to 3 years They further showed the occurrence of *H influenzae* meningitis to be inversely releated to the presence of bactericidal activity in the same age groups The bactericidal activity demonstrated was later on interpreted as an activity dependent on the presence of capsular b antibodies (20) Recent studies concerning the bactericidal activity of sera from individuals immunized with purified capsular substance b tend to support this interpretation (2 17)

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THE RELEVANCE OF A SEROLOGICAL CLASSIFICATION OF CHRONIC HEPATITIS

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Forty two consecutive patients with biopsy verified chronic hepatitis and available serum specimens were examined for the occurrence of Australia antigen, antinuclear antibodies, smooth muscle antibodies and mitochondrial antibodies Twelve patients with persistent Australia antigen, but without circulating autoantibodies (group 1) were compared with twenty seven patients without Australia antigen, but with circulating autoantibodies (group 2) Young males with a history of serum hepatitis were predominant in group 1, while most of the patients in group 2 were middle aged women with an insidious onset of the disease The biochemical activity of the liver disease was more pronounced in group 2 It is concluded, that the histological defined chronic hepatitis may include several disease entities of which at least two can be separated by the serology The relevance of this classification is supported by the fact that there are more patients developing cirrhosis in group 2 than in group 1

Chronic hepatitis as defined by *De Groote et al* (5) falls in two groups chronic persistent hepatitis and chronic aggressive hepatitis While the prognosis for patients with chronic persistent hepatitis is regarded good (1), and progression to cirrhosis is an exception, patients with chronic aggressive hepatitis often develop cirrhosis within a short period of time (4)

Recently *Christoffersen et al* (4) demonstrated a classification of chronic aggressive hepatitis on basis of the occurrence of abnormal bile duct epithelium into two groups with different prognosis

Wright (14), and later *Vischer* (11), and *Bulkley et al* (3) offered the possibility of classifying patients with chronic hepatitis into serological homogenous groups, finding a mutal exclusion between circulating autoantibodies and Australia antigen, and they suggested a common pathogenesis within the groups

The aim of the present study was to explore the clinical relevance of this serological classification in a group of patients with well-defined histological proven chronic hepatitis

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MATERIAL AND METHODS

The material comprises 42 consecutive patients with biopsy verified chronic hepatitis and available serum specimens admitted to the Second

Medical Department, Kommunehospitalet and the University Clinic for Infectious Diseases Blegdam Hospital Copenhagen

In all cases the histological examination of the liver biopsy was performed by the same pathologist (PC) without knowledge of the clinical diagnosis All patients fullfilled the criteria put forward by *De Groote et al* (5)

From all patients exist serum specimens from the time of biopsy, which were examined for the presence of Australia (Au) antigen antinuclear antibodies (ANA), smooth muscle antibodies (SMA), mitochondrial antibodies (MA), and concentration of immunoglobulins

For demonstration of Au antigen, an immunoelectroosmophoresis according to the technique of *Prince & Burke* (9) was employed All positive reacting serum specimens were tested for identity by immunodiffusion with known reference serums The antiserum against Au antigen was derived from a patient with aplastic anemia and showed identity with anti Au serums from other centers (7)

ANA and MA were determined on human thyroid liver and kidney tissue by the indirect immunofluorescence technique, using fluorescein iso-thiocyanat labelled immunoglobulins against IgG and IgM The specificity of the used antibodies was controlled by immunoelectrophoresis (4) Serum specimens were tested undiluted

SMA was determined in serum specimens diluted 1 20 with human uterus tissue from an 0 rhesus negative person as antigen using the above mentioned conjugate against IgG Healthy blood donors of the same sex and age as the patients in the material were included as controls

Laboratory examinations were made by conventional methods Fractionation of serum proteins was made by paper electrophoresis and quantitation of IgA IgM, and IgG was performed by electrophoresis in antibody containing gel as described by *Weeke* (12)

On all but three patients repeat biopsies have been performed These biopsies have been assessed to the same criteria as the primary biopsies The average period of observation (time between diagnostic biopsy and follow up biopsy) was 23 months varying from one month to 45 months

Twelve patients were treated with steroids five with 6 mercaptopurine and four with both steroids and 6 mercaptopurine

For the statistical assessment the chi test and Student s t test have been used The limit for type 1 error (2 alfa) has been set to 0 05

TABLE 1 *Serological Tests in 42 Patients with Chronic Hepatitis*

Total patients	42
Serological tests	
Australia antigen (Au antigen)	13
Antinuclear antibodies (ANA)	21
Smooth muscle antibodies (SMA)	20
Mitochondrial antibodies (MA)	0

TABLE 2 *Correlation between Australia Antigen (Au) and Antinuclear Antibodies (ANA) and Smooth Muscle Antibodies (SMA) in 42 Patients with Chronic Hepatitis*

Serological results	Au (+) 13 patients	Au (—) 29 patients
ANA (+)	0	21
ANA (—)	13	8
SMA (+)	1	19
SMA (—)	12	10

RESULTS

Serological Findings

The presence of Au antigen and circulating autoantibodies are seen from Table 1 Thirteen patients were positive for Au antigen while ANA and SMA were found in 21 and 20 patients respectively MA could not be demonstrated in any of the patients in this study

Table 2 gives the correlation between Au antigen and circulating autoantibodies (ANA and SMA) None of the patients had both Au antigen and ANA, and only one had simultaneous occurrence of Au antigen and SMA (in low titre) ANA and/or SMA were demonstrated in 27 patients without Au antigen, and two patients had neither Au antigen nor ANA or SMA

TABLE 3 *Histological Aspects in Group 1 (12 Patients with Au antigen) and Group 2 (27 Patients with ANA and/or SMA)*

	Group 1	Group 2
Histological diagnosis		
Chronic aggressive hepatitis	9	24
Chronic persistent hepatitis	3	3

On the basis of these serological results two groups were formed *Group 1* including 12 patients with Au antigen, but neither ANA nor SMA, and group 2 consisting of 27 pa tients without Au antigen, but with ANA and/or SMA

The histological, clinical, biochemical, and prognostic features in the two groups have been compared

Histological Findings

Group 1 comprises nine patients with chro nic aggressive hepatitis and three patients with chronic persistent hepatitis, and *group 2* comprises 24 patients with chronic aggressive hepatitis and three patients with chronic per sistent hepatitis (Table 3) There is no significant difference between the two groups

Clinical Findings

Age and sex distribution in the two groups are seen from Table 4

In *group 1* there are ten men and two women and the average age is 23 years Group 2 consists of four men and 23 women with an average age of 58 years There are significantly more women in group 2 than in group 1 ($p<0\,001$), and the mean age is significantly higher in the same group ($p<0\,001$)

As seen from Table 5 the onset of the ill ness was acute (debuting as a clinical acute viral hepatitis) in all 12 cases from group 1, and in nine cases from group 2 In all but one case a biopsy fullfilling the histological criteria of acute viral hepatitis (2) has been obtained Statistically there are significantly more cases with insidious onset of illness in group 2 than in group 1 ($p<0\,05$)

TABLE 4 *Age and Sex Distribution in Group 1 (12 Patients with Au antigen) and Group 2 (27 Patients with ANA and/or SMA)*

	Group 1	Group 2
Sex		
Male	10	4
Female	2	23
Age mean (year)	23 2	58 4
	(16-44)	(15-82)

TABLE 5 *Clinical Aspects in Group 1 (12 Patients with Au antigen) and Group 2 (27 Patients with ANA and/or SMA)*

	Group 1	Group 2
Type of onset		
Abrupt	12	9
Insidious	0	18
Exposure		
Infectious hepatitis	3	9
Serum hepatitis	9	0
Abuse of narcotics	8	0

Nine of the patients in group 1 with abrupt onset had a history of serum hepatitis, and eight of them were drug addicts In group 2 all nine patients with acute onset had a history of epidemic or infectious hepatitis

Biochemical Findings

The average values for some conventional liver tests can be seen in Table 6

The activity of the disease was more pronounced in group 2 than in group 1, but the scatter of the values for serum bilirubin tran saminases and alkaline—phosphatases within each group is too great to allow a statistical comparison

There are significantly more patients with hypoalbuminemia in group 2 than in group 1, and the value of IgA and IgG were sig nificantly higher in the same group ($p<0\,01$) (Table 6 and Fig 1)

Follow up

Table 7 shows the result of the morfologi cal follow up of all 12 patients in group 1 and of 24 patients in group 2 The time of observation is 19 and 25 months respectively

One patient from group 1 developed cirrhosis, (originally chronic aggressive hepatitis), and the rest still shows chronic hepatitis with varied degree of activity None of the patients in this group died during the time of observation

Seven cases from group 2 developed cirrho-

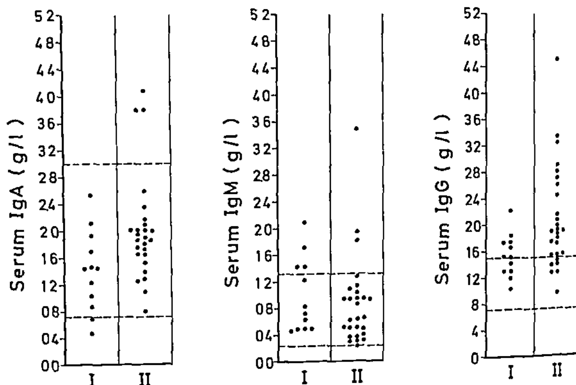


Fig 1 Serum immunoglobulins in chronic hepatitis associated with Australia antigen (I) and circulating autoantibodies (II)

sis (originally chronic aggressive hepatitis), and two have died during steroid therapy In both cases autopsy revealed chronic aggressive hepatitis The remainder 15 patients in this group still have chronic hepatitis in the follow-up biopsies

There are significantly more patients developing cirrhosis in group 2 than in group 1 ($p < 0\ 001$)

None of the patients in group 1 have been treated with steroid or other immunosuppressive drugs In group 2 twelve patients go

TABLE 6 *Average of Liver Tests in Group 1 (12 Patients with Au antigen) and Group 2 (27 Patients with ANA and/or SMA) Normal Values are Given in Parentheses below the Name of the Laboratory Test*

	Group 1	Group 2
Serum bilirubin (<1 0 mg/ml)	0 8 (0 3–1 0)	3 2 (0 5–13 8)
Serum GO transaminase (<34 u/l)	117 (21–230)	350 (12–1200)
Alkaline phosphatase (<74 u/l)	92 (41–186)	179 (45–675)
IgA (0 74 3 06)	1 40 (0 69–2 54)	2 03 (0 79–4 14)
IgM (0 23 1 33)	1 01 0 50–2 11)	0 88 (0 24–3 50)
IgG (7 2 15 1)	15 6 (10 7–22 8)	20 8 (9 9–45 4)
No of patients with hypoalbuminaemia	1	15

TABLE 7 *Follow up of Group 1 (12 Patients with Au antigen) and Group 2 (27 Patients with ANA and/or SMA)*

	Group 1	Group 2
Time of observation		
Mean (months)	19	25
Histological remission	0	0
Cirrhosis hepatitis	1	7
Dead	0	2

steroid therapy, five patients 6 mercaptopurine, and four patients have been treated with both steroids and 6 mercaptopurine

DISCUSSION

The total incidence of Au antigenemia in the present material is 31 per cent, and is in accordance with previous reports (3 11 13) from Switzerland, Boston, and Yale

Our serological results confirms that of *Bulkley et al* (3) in respect that none of the Au antigen positive sera contained ANA as well, and the lack of association between Au antigen and SMA is in agreement with others (10 11 14)

Doniach et al (6) found the SMA test positive in serum of 67 per cent of patients with classic active chronic hepatitis, which corresponds to our findings in the Au-antigen negative group

The comparative study between the group with Au antigen but without ANA and SMA (group 1) and the group without Au antigen but with either ANA or SMA (group 2) reveals several significant differences in clinical and prognostic parameters Most of the patients in group 1 are young males with abrupt onset of the illness while group 2 mainly consists of middle aged women with insidious onset of the disease Further, the biochemical activity is more pronounced and

The observations suggest at least two different types of chronic hepatitis, and may reflect aetiologic differences between the two groups One type caused by a persistent viral infection (8), with a mild course and rather good prognosis Another type may be a true autoimmune disease caused by unknown factors leading to a self perpetuating liver-cell injury, with an active course of the disease and a bad prognosis

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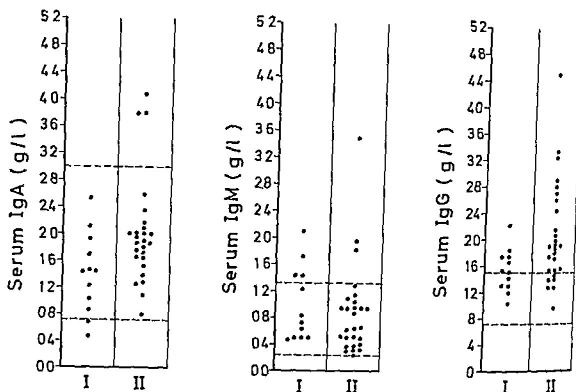


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IgG (7 2 15 1)	15 6 (10 7–22 8)	20 8 (9 9–45 4)
No of patients with hypoalbuminaemia	1	15

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ELECTRON MICROSCOPICAL AND CULTURAL FEATURES OF *NEISSERIA MENINGITIDIS* COMPETENCE VARIANTS

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Competent and incompetent variants of five strains of *Neisseria meningitidis* were examined by negative stain electron microscopy with the aim of detecting a possible correlation between the competent state in DNA mediated transformation and the presence of special surface structures. As previously reported for species of *Moraxella*, a correlation between the occurrence of fimbriae at or near the cells and a high level of competence was found. Various conditions of cultivation were investigated to see if small, previously unnoticed, differences in type of growth could exist between the competent and incompetent variants. No difference could be detected in colony morphology on solid medium. However, in statically incubated broth the competent variants were distinguished by forming a surface pellicle after one to two days of growth.

Previous studies have demonstrated that several species and strains of the genus *Moraxella* regularly produce variants distinguishable by their different colony morphology (8, 13, 17). By cultivation on blood agar in a humid atmosphere one can define spreading and corroding colony forms (SC type) and a non-spreading and non corroding type (N). Cells from the SC type of colony are by electron microscopy found to be fimbriated. The N type of colony consists of cells having none or only few and late appearing fimbriae (6, 8, 13). In statically incubated broth culture the SC variants usually form a surface pellicle.

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Bidirectional spontaneous variation between the two colonial types of *Moraxella* occurs at a low frequency. Generally, the SC type is most often found in recently isolated strains, and the N type tends to predominate after subcultures on solid medium (5, 6, 8, 13, 37).

When the two types of colony variants of *Moraxella bovis*, *M. kingii* and *M. nonliquefaciens* are tested as recipients in homologous transformation to streptomycin resistance, the fimbriated (SC) variants are found to yield from about 10^{2} fold to about 10^{4} fold more transformants per exposed colony forming unit than the nonfimbriated (N) variants (7, 8, 9).

Neisseria meningitidis has regularly been shown to be highly competent in genetic transformation tests shortly after isolation from clinical material (32). With a varying

frequency, however, the different strains dissociate a proportion of colonies with lost (or very much reduced) competence In two meningococcal strains more thoroughly studied (M1 and Ne 15), competence has been found to depend upon a heritable factor coined *cp* (23, 25) This factor shows several features that are generally ascribed to episomal structures (23, 26, 29) At least in the strain M1 it seems that a permanent loss of the factor is strictly correlated with a complete loss of the ability to take up extracellular DNA (16, 31)

It is well known from work with fimbriae in enterobacteria that the genetic determinants of such structures may reside in episomal or cytoplasmic elements In view of the findings in *Moraxella* it was accordingly decided to examine several pairs of competence variants of *N meningitidis* for the presence of fimbriae A search was also started for a difference in the type of growth on solid and fluid media similar to that observed in *Moraxella*

MATERIALS AND METHODS

Bacterial Strains

Five strains of *N meningitidis* were examined They had all been isolated from cerebrospinal fluid or blood in cases of meningitis The strains M1 (22), B8152/66 (4, 30), Mad 1 and P22 (19) were all from patients in Norway, whereas the strain Ne 15 originated from a case of fatal (non epidemic) meningitis in USA (10) The strains Mad 1 and P22 were isolated as sulfonamide resistant variants (19) and strain B8152/66 as a non saccharolytic one (4 30) The other strains had the ordinary wild type' characteristics of *N meningitidis* but in this work the auxotrophic mutant M1 8 *arg his* of strain M1 was used (25)

Genetic Procedures

DNA preparation and transformation procedures followed the previously described technique (27) Auxotrophic mutants of the strain M1 as well as the streptomycin resistant mutant (Str r) of this strain used for the production of transforming DNA, were the same as those previously used (25) Genetically competent [illegible]ants indicated by the symbol cp^{+} and genetic[illegible]ncompetent ones indicated by *cp* were contr[illegible]ed as described before (23, 25) Genetically incompetent variants were obtained by monoclonal isolation either after spontaneous loss of competence (32), or after treatment with ethidium bromide (26) Transformation and mutation frequencies in the *str* locus were calculated as the ratio of the number of colonies found on streptomycin plates to the total number of colony forming units obtained when plated on complete agar medium devoid of streptomycin (24)

Electron Microscopical Technique

Bacteria growing semiconfluently on human blood agar at 33° C in a humid atmosphere for 5 to 25 h were collected and prepared for electron microscopy as described for *Moraxella* (8) For most of the present study 0 8 per cent (w/v) *sodium silicotungstate (pH 6 5)* was employed as stain and occasionally 2 5 per cent (w/v) ammonium molybdate (pH 7)

Several hundred cells were studied in each preparation Generally, most of these happened to be in aggregates, but at least ten per cent single cells (or two- to four-cell aggregates) were included in a search

Cultural Conditions for Study of Growth Characteristics

Colony appearance was studied on human blood agar after incubation in a very humid atmosphere (no CO_2 added) as in the study of *Moraxella (8)* Growth in statically incubated Mueller Hinton Broth (Difco) was examined under conditions identical to those employed for the latter organisms (8) The incubation temperature was usually 33°C with some comparative experiments performed at 37° C

RESULTS

Transformability

Transformation as well as spontaneous mutation in the *str* locus were examined in the five *N meningitidis* strains When subjected to the same, standard technique the competent variants of the strains M1 and P22 constantly gave transformation frequencies a little lower than those observed in the other strains (Table 1) The incompetent variants were further checked No experiment showed significantly higher frequency of Str r clones with Str r *N meningitidis* DNA than what was obtained in control systems with no DNA, with salmon sperm DNA, or with *Escherichia coli* Str r DNA

TABLE 1 *Fimbriation and Pellicle Formation in Competence Variants of* N *meningitidis*

Strain	Sero group	Variant	Control of competence* Transformation frequency	Mutation frequency	Degree of fimbria-tion**	Pellicle forma tion***
Mad 1	A	cp^{+}	$1\ 35 \times 10^{3}$	$<1\ 07 \times 10^{7}$	100%	+ + 1
Mad 1	A	*cp*	$<1\ 11 \times 10^{7}$	$<1\ 15 \times 10^{7}$	<1%	—4
B8152/66	A	cp^{+}	$1\ 36 \times 10^{3}$	$7\ 25 \times 10^{8}$	50–100%	+ 1
B8152/66	A	*cp*	$<1\ 66 \times 10$	$<1\ 44 \times 10^{7}$	<1%	—4
Ne 15	B	cp^{+}	$1\ 44 \times 10^{3}$	$4\ 32 \times 10^{-8}$	3–7%	+ + 1
Ne 15	B	*cp*	$<1\ 39 \times 10$	$<1\ 21 \times 10^{7}$	<1%	—†
M1 8 *arg his*	B	cp^{+}	$4\ 89 \times 10^{4}$	$7\ 95 \times 10^{-8}$	10–30%	+ + 1
M1 8 *arg his*	B	*cp*	$1\ 23 \times 10^{8}$	$<9\ 64 \times 10^{8}$	<1%	—4
P22	C	cp^{+}	$5\ 95 \times 10^{4}$	$<1\ 41 \times 10^{7}$	2%	+ 2
P22	C	*cp*	$<1\ 48 \times 10^{7}$	$<1\ 97 \times 10^{7}$	<1%	—4

* Transformation was performed with DNA from a streptomycin resistant mutant of the strain M1 as described (27) Mutation frequency was measured with salmon sperm DNA instead of *N meningitidis* DNA Frequencies are given as the ratio of the number of colonies found on streptomycin plates to the total number of colony forming units obtained on complete agar medium devoid of streptomycin (24)
** Semiquantitative estimates by electron microscopy of negative stain preparations from 5 9 h old co lonies The approximate percentage of cells carrying at least one fimbria are given The low levels were difficult to estimate more accurately than to less than one per cent The actual counts are given in the text as well as a discussion of problems in connection with their assay
*** An inoculum was incubated statically in tubes with Mueller Hinton Broth (Difco) at 33° C (see the text) + + heavy pellicle formation + clearly detectable pellicle The day of appearance is given —4 no trace of pellicle detected during four days of incubation —† a trace of clear mucous material detected at the meniscus after about three days, see the text for details

The loss of competence was also examined In the strain M1, spontaneous loss was observed in 0 5 to 2 per cent of the clones which appeared upon spreading on blood agar plates, with some variation between individual experiments In the other strains, spontaneous loss was less than 0 4 per cent These four strains are thus more stable with regard to competence than most patient strains previously examined (32) In all the strains the loss of competence could be strongly increased by treatment with ethidium bromide (26)

Electron Microscopy

By electron microscopy of negatively stained cells, harvested from the agar surface after different periods of growth it was easily detected that the competent variants were fimbriated to a m 1 ~ e er extent than the incompetent or s T ' le 1) The fimbriae were similar to r *Moraxella* (6, 8, 13) and to the type-1 fimbriae of enterobacteria (3) in diameter and general appearance Both types of variants of all strains grew as diplococci of about one μm in diameter Fig 1 shows the cp^{+} variant of strain Mad 1 and Fig 2 the *cp* variant, both prepared after 7 h of incubation, demonstrating clearly that the cp^{+} cell has several long filamentous appendages whereas the cp^{-} carries none The degree of fimbriation of cp^{+} and the difference between the variants were not, however, always as marked as in this strain Each strain seemed to have distinct features which will be described in the following The main results are summarized in Table 1

Usually it was easier to study the cells grown for the shorter times (5 to 9 h) because the preparations then were "cleaner" (see below about cell wall extensions) Once, however, it was difficult to study bacteria grown for 7 h This was probably the result of very little growth due to more than usually dry


2
e


3
4
r

media With *N meningitidis* B8152/66 cp^+ the fimbriation was definitely more pronounced after the shorter incubation times (see below) In the other strains it was impossible to see any clear variation with period of growth

Mad 1 After 7 and 9 h growth almost every cp^+ cell showed several straight or just slightly curved fimbriae of 60 70 Å diameter The length varied from ½ μm to several μm (more than 10 μm were found occasionally) The fimbriae were usually single with little tendency to attach side to side There seemed to be no distinctly preferred site of origin on the bacterial surface (Fig 1) At the same time none of the *cp* cells disclosed any typical fimbriae although several hundreds were scrutinized (Fig 2) A few possibly atypical fimbriae were detected at the level of 1 per 80 100 cells (Fig 4) The cp^+ cells at 7 and 9 h and the *cp* cells at 9 h were surrounded by structures which gave the appearance of tubular extensions of the bacterial surface layers Similar structures in *Moraxella* have been described as cell wall extensions (14) Presumed fragments of such extensions were also apparent (in several cases as the only type seen around many *cp* cells at 7 h) (Fig 2) Fig 3 demonstrates that also the *cp* cells at 7 h may have tubular cell wall extensions

B8152/66 At 6 h almost every cp^+ cell of this strain was richly fimbriated (Fig 5), occasionally with many filaments in one or more tight bundles of considerable length (Fig 6) At 9 h the thick bundles were rarer to find but the cells were still almost all fimbriated As a rule several fimbriae emerged from each diplococcus in all directions At 12 h the percentage of fimbriated cells had dropped to about 2 and bundles were inapparent The *cp* cells were at all times devoid of fimbriae (none were found among 500 cells studied) In this strain especially, the *cp* cells were characterized by a densely granulated region surrounding the cells (Fig 7) Both cp^+ and *cp* cells had cell wall extensions similar to those seen in Mad 1 Also here they were usually evident as fragments near *cp* cells at 6 h (Fig 7)

Ne 15 is a representative of a pair with few fimbriated cells in the cp^+ population At 5½ to 25 h only 3 to 7 per cent of the cp^+ cells carried one fimbria (Fig 8) The fimbriae were usually 1 to 3 μm long or less and not quite as straight as in the strains above They even had sharp bends in a few instances (not shown) The fimbriae seemed more distinct after 5½ h than later More than 400 cells of the corresponding *cp* variant were scrutinized without finding traces of fimbriae (Fig 9) In another search 300 cells were carefully observed after 5½ and 8 h growth without finding a single one fimbriated However, a group of fimbriae (not shown) without connection to any of the cells in the same

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Fig 1 4 Negative Stain Preparations of Neisseria meningitidis Mad 1 Variants

See the text for methods Stain 0 8 per cent sodium silicotungstate (pH 6 5) To see details also in the darker areas of the prints light areas of the negative were often given a reduced exposure in printing The bar marker in this and subsequent figures represents 0 1 μm Magnification 1 and 2 40 000 1 3 and 4 30 000 1

Fig 1 Mad 1 *cp* , 7 h growth Fimbriae (f) emerging from the cell in several directions only slight side-to-side association (▲) is seen Cell wall extensions (e) also from several regions Tubular appearance near the cell with widened portions peripherally Presumed fragments (o) of wall material are seen in more or less close association with the cell wall extensions The light area of varying width (r) circumscribing most of the cell represents a defect in the negative stain film

Fig 2 Mad 1 *cp* , 7 h growth with short cell wall extensions (e) and fragmented material (o) thought to be derived from these No fimbriae detectable

Fig 3 Mad 1 *cp* , 7 h growth A few cells featuring the presence of tubular cell wall extensions (e) with dilated regions Globular elements (o) are also seen The rifts in the negative stain film (r) in many places occur corresponding to tubular structures (x)

Fig 4 Mad 1 *cp* 7 h growth with dense accumulation of stain near a group of cells (not shown) and a wide rift (r) The arrow points at a structure which probably is not a piece of a fimbria but seems to resemble one

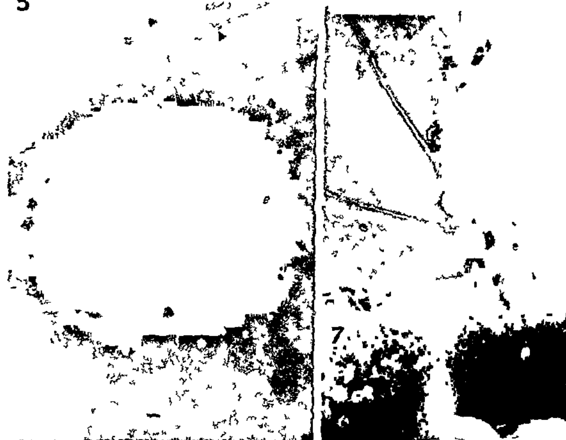


Fig 5 7 N men ngitid s *B8152/66 l ariants* See the text for methods Stain 0 8 per cent sod um s lico-tungstate Magnif cation 30 000 1

Fig 5 B8152/66 cp 6 h growth Cell wall extensions (e) of varied form w th fragments (o) accumu lation of granular material *near the cell* bodies and a number of f mbr ae (f) seemingly rad at ng f om the d plococcus are demonstrated A fimbria is pass ng the edge of one cell Ma ked tendency for side to s de pc sit oning of pa rs if fimbriae (▲) is seen

Fig 6 B8152/66 *cp* 6 h *growth Bundles* of f mbriae cross ng each other Also a few s ngle fimbriae (f) are seen as well as a long p ece of tubule l ke cell wall extension (e)

Fig 7 B8152/66 cp 6 *h growth* Accumulation of large amounts of granular mater al around cells together with presumed fragments (o) of cell wall extensions are seen

preparation was found in this search Also in this strain the cells of both variants in a very comparable manner at all times were surrounded by num°rous extensions from cell surfaces They were of a smaller diameter than the majority of those seen with the above mentioned strains These cells were not embedded in densely granulated material in any of the preparations investigated See be low for additional experiments with this strain

M1 8 arg his This strain showed f mbria tion of about 20 per cent of the *cp* cells after 7 (very little growth due to unfavourable con ditions) and 9 h of cultivation These fim briae were often found in aggregates of 2 to 10 units somewhat loosely arranged in bund les The length was mostly l mited to 1 to 3 µm Centre to-centre measurements of paral lel running fimbriae ind cated a diameter of about 65 Å (Fig 11) Also in this strain the

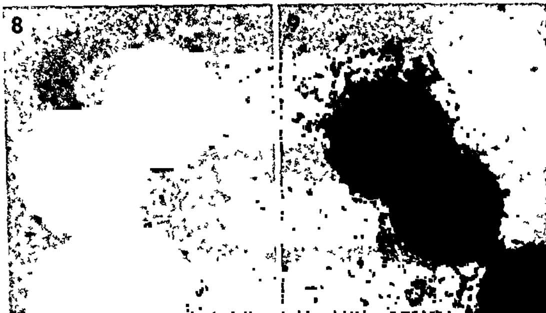


Fig 8 and 9 N meningitidis *Ne 15 Variants* See the text for methods Stain 0 8 per cent sodium silicotungstate Magnification 30 000 : 1

Fig 8 Ne 15 *cp*⁺, 5½ h growth A group of cells with two single fimbriae (f) is seen The cell wall extensions (e) seem more delicate than in previous illustrations and occasionally disappear in the granular background structure Dilated portions and presumed fragments (o) are also here demonstrable

Fig 9 Ne 15 *cp*⁻, 6 h growth Very similar to the *cp*⁺ variant except for the lack of fimbriae

tubular material around the cells was of a delicate nature and often seemed disrupted (Fig 10) The fimbriae were occasionally associated with such tubular or granular material and appeared more indistinct in the preparations (Fig 12) The *cp*⁻ variant was non fimbriated at the level of 300 cells scrutinized The extracellular material often appeared in large masses of a very varied morphology (Fig 13)

P22 This was the strain of those here studied, with the seemingly least fimbriated *cp*⁺ variant Only 2 per cent of the *cp*⁺ cells carried fimbriae detectably These fimbriae were often short (0 3 to 0 5 μm) and somewhat indistinct, but long (2 to 3 μm) and distinct ones were also evident (Fig 14) Fimbriae were only found with aggregated cells in this strain The *cp*⁻ cells were generally non fimbriated (Fig 15), but after having scrutinized altogether more than 600 cells of this variant, grown for 7 h, a group of distinct and about 1 μm long fimbriae were detected (Fig 16) Also one questionable (short, indistinct) fimbria (not shown) was found in this search of the *cp*⁻ The cell wall extensions were quite predominant in this strain (resembling Mad 1 and B8152/66 in this respect) and were found as thin and thick tubules with dilated portions in addition to considerable amounts of fragmented material There were also often large collections of granular material close to the cells of both variants at early as well as at late times (not shown)

Colony Morphology on Solid Medium

Distinct agar corrosion underneath scraped off colonies was not observed in *N meningitidis,* irrespective of variant type The standard cultural conditions referred to in Materials and Methods were modified by using higher and lower contents of agar in the medium (varying from 0 6 to 2 4 per cent) and by increasing the incubation tem-

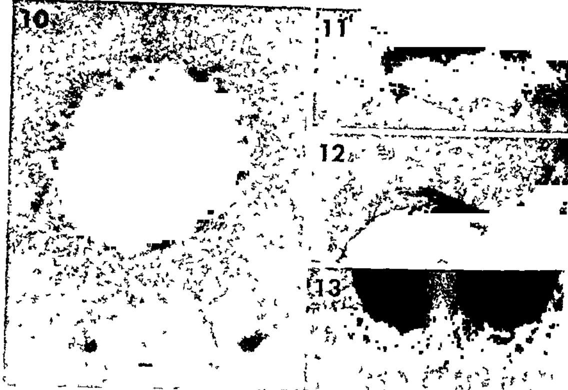


Fig 10 13 N meningitidis *M1 8* arg his *Variants* See the text for methods Stain Fig 11, 2 5 per cent ammonium molybdate, the others, 0 8 per cent sodium silicotungstate Magnification 30 000 1

Fig 10 M1 8 *arg his cp*⁺, 9 h growth Typical cell with one fimbria and a varied array of cell wall extensions close to the cell body

Fig 11 M1 8 *arg his cp*⁺, 12 h growth. Part of a cell stained with ammonium molybdate A bundle of aggregated fimbriae and a short single fimbria (arrow), seemingly pointing out of the cell are seen

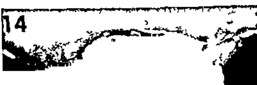
Fig 12 M1 8 *arg his cp*⁺, 12 h growth Groups of fimbriae with associated extracellular material are seen

Fig 13 M1 8 *arg his cp* , 9 h growth Part of two cells with extracellular structures but no fimbriae is seen

perature from the usual 33° C to 37° C, without change in this respect Neither were spreading of the colonies or other particular morphological traits of the variants observed Thus, fimbriation and competence do not provide *N meningitidis* with a clearly distinguishing colony type on solid medium under the conditions investigated, contrary to the case with *Moraxella* (8, 9, 13, 37)

Growth Characteristics in Fluid Medium

In all five *N meningitidis* strains examined, the *cp*⁺ variant formed a surface pellicle after static incubation of fluid cultures for 1 to 2 days (Table 1) The pellicle formation appeared to be favoured by incubation at 33° C as compared to 37° C The surface growth film was loose, being easily disturbed by the slightest agitation of the tube The most conspicuous indication of surface growth was an opaque rim sticking to the glass wall In all strains except Ne 15 no indication of surface pellicle or rim was found in the *cp* variant during 4 days of incubation In Ne 15 *cp* , after about three days of incubation, a trace of clear mucous material was found sticking to the glass wall at the broth surface This rim did not develop further and was distinct from the opaque pellicle of the *cp*⁺ variant Thus, the fimbriated, compe





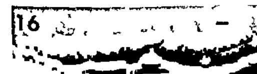


Fig 14 16 N men ngit d s *P22 Variants* See the text for methods Stain 0 8 per cent sodium s l co tungstate Magn f cat on 30 000 1

Fig 14 P22 *cp* 7 h growth Chain like cell aggre gate with one fimbriae (f) Cell v all extens ons (e) are oriented along the aggregate

Fig 15 P22 *cp* 7 h growth Very s m lar to *cp* except for lack of f mbriae Fragmentat on of cell wall extensions

Fig 16 P22 *cp* 7 h growth A group of short f m briae (f) separated from the cells by a rift (r) in the negat ve stain film

tent type of *N meningitidis* shares the distin guishing characteristics of fimbriated compe tent moraxellae in statically incubated fluid cultures (6 8 9 13)

Additional Experiments

After having completed the initial phase of these investigations continued studies of cells derived from surface pellicles in static broth cultures were undertaken In the strain Ne 15 cells grown from the indistinct sur face rim formed by the *cp* variant were uni formly low or incompetent as shown by secondary streaking on DNA supplemented blood agar (23) One of the colonies was exa m ned b ele

ly competent by the same test On one single occasion however one out of eight colonies from such a cp^{+} pellicle was found to contain low or incompetent cells This new variant was shown to be distinctly more fimbriated than the Ne 15 cp^{+} parent type (see above) 35 to 60 per cent of the cells each had one or two fimbriae, and occasionally many fimbriae were found with a small group of cells a high competent clone isolated from the very same pellicle had as usual for Ne 15 cp^{+}, only about 5 per cent fimbriated cells The gross morphology of the fimbriae was not distin guishable between the two cases This low or incompetent but highly fimbriated variant of *N meningitidis* Ne 15 cp^{+} appeared even more rapidly pellicle forming than its parent In a repeated experiment including 100 colo nies derived from a Ne 15 *cp* pellicle all cells were highly competent however

DISCUSSION

Competence Variation and Fimbriation

It seems that fimbriae are present in all *cp* variants of *N* m

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phenotype with no or extremely few fimbriae With a view to the previous observations in

N meningitidis regarding this particular type of loss of genetic competence (26), the present findings may indicate that the genetic determinant for the fimbriae specifically observed in the competent genotype actually resides in the (episomal) *cp* factor But this does not necessarily mean that the presence of fimbriae must inevitably be followed by a competent genotype Since it is known that bacteria may possess several types of fimbriae at the same time (3), we must expect also to find strains of *N meningitidis* carrying more than one type of fimbriae This may be the reason why in a few instances (P22, Fig 16) the cp^- genotype was found to contain occasional fimbriae in the preparations although an infrequent genetic variation from non fimbriation to fimbriation cannot be excluded In one instance, a clearly more fimbriated variant from the otherwise sparingly fimbriated Ne 15 cp^+ turned out to be incompetent But it is remarkable that this variant was selected by growth in the surface pellicle of statically incubated cultures conditions which favour the selection of fimbriated cells in *E coli* (3) as well as in *Moraxella* (8), whereas the loss of competence was a scored property Possibly the parent strain Ne 15 cp^+ carries two or more types of fimbriae, the one being determined by genes residing in the *cp* factor If this factor determines the synthesis of a repressor like the fi^+ type of R factor (12), the selective system used could actually screen for those variants having lost the *cp* factor, while preserving the determinant for the other type(s) of fimbriae

DNA transformation is a complex process, and impaired competence may of course also be of a nature entirely different from that consistently found in the strain M1 after spontaneous or ethidium bromide induced loss (16 31) Various types of competence mutants have indeed been described (38) In the *N meningitidis* strain M1 (24) as well as in *Bacillus subtilis* (18) a drastic reduction of the transformation frequency has for instance, been observed in UV sensitive mutants that were (presumably) defective in the recombination system (*rec*) The *N meningitidis* mutants of this genotype still contain the *cp* factor (24) If the genetic determinants for fimbriae are carried by the factor the cells should thus be of a fimbriated phenotype

The number of fimbriae on each cell and thereby probably also the number of detectably fimbriated cells may change with the environment, as well as with the metabolic situation bearing no strictly fixed relation to the genotype (11, 33 36) The very distinct decrease in fimbriation of B8152/66 *cp* from 6 to 12 h of growth represents such an example One must consider mechanisms of fimbrial removal (breakdown) and suppression of their formation as well as selective intracolonial growth of non fimbriated cells

The possible existence of a mechanism with alternating extrusion and withdrawal of fimbriae (2, 20, 33, 34) points to problems when trying to estimate their number Obviously the actual counts only express the situation at one particular moment Mechanisms by which fimbriae are arrested in extruded form may be most important It is conceivable that aggregated fimbriae can no longer retract in analogy with the situation found after phage (2) and antibody absorption (33)

For these reasons, it is difficult to make assumptions on the possible role of fimbriae based on sligth differences in fimbriation such as degree of competence in different *cp* variants in relation to percentage of fimbriated cells (Table 1) It should be pointed out that the electron microscopical examinations in this study were performed under growth conditions differing from those used during the quantitative short term DNA exposure transformation experiments However we feel that the considerable difference in fimbriation generally found between cp^+ and *cp* variants is significant and largely corresponds to the previously reported findings in *Moraxella* (9), where fimbriated variants are distinctly more competent in genetic transformation than their non fimbriated counterparts The same seems to be the case in *N gonorrhoeae* (21, 39 40) The mechanism by

which fimbriae could possibly be actively involved in the transformation process, as well as the possibility that they are functionally inert, have been briefly discussed previously (9, 15)

Other Correlates to Fimbriation

The proportion of fimbriated cells in *cp* populations of *N meningitidis* appears unrelated to the frequency of the loss of competence (Table 1 and the text) But a correlation may exist between the degree of fimbriation and the serological group in the way that strains belonging to group A are heavily fimbriated, those of group B much less so, whereas the group C strain examined is very sparingly fimbriated (Table 1) A conclusion with regard to this possibility would, naturally, require substantial elaboration

In *Moraxella bovis*, the presence of fimbriae has recently been shown to be correlated to the ability of colonizing the bovine conjunctiva and eliciting keratoconjunctivitis (37) In *N gonorrhoeae*, it appears essential for virulence that the bacteria are of the fimbriated type (40) Therefore, it is of interest that competence in transformation, which now seems constantly to be followed by fimbriation is always present in *N meningitidis* strains isolated from the cerebrospinal fluid or blood of patients, although the property may be lost from all strains (28, 32)

For further investigations on the relations between fimbriation and competence/parasitism/virulence it is important to note that the cultural correlates to fimbriation may differ between species Thus only part of the cultural behavior pattern of the fimbriated *Moraxella* variants (agar corrosion, spreading surface pellicle formation) is shown by fimbriated *N meningitidis* (pellicle formation) In *N gonorrhoeae*, there are still other cultural hallmarks of fimbriation (21, 39, 40)

Extracellular Structures besides Fimbriae

The present methods of preparing cells for electron microscopy are quick and should be considered relatively mild towards the cells because so few manipulations are involved Some of the extracellular structures give the appearance of being membraneous tubules which on occasions can be found to arise from the outer leaflet of the cell wall (not shown here) The fact that two different negative stain solutions of 45 fold different osmolality (35), give rise to essentially similar structures may indicate that the cell wall extensions are not an artifact of the osmotic difference between the cells and the stain solution It must be admitted, however, that every preparation goes through the stage of a saturated solution before electron microscopy Such structures have previously been detected by similar as well as other methods in organisms like *Bdellovibrio, Bordetella, Moraxella* (14), *Escherichia coli* (1), and *N gonorrhoeae* (41)

Liquid cultures of *N meningitidis* have recently been investigated by thin sectioning for electron microscopy of antigenic material obtained from the broth and by extracting the cells A cell wall complex has been isolated and characterized and it is proposed to be representative of the outer trilaminar membrane of the meningococcal cell envelope in its native state (42)

The granular material described around some of the cells of *N meningitidis*, particularly *cp*, is of completely unknown nature and origin

The extracellular structures besides fimbriae represent a most conspicuous feature of the meningococci grown on agar The cell wall extensions display similarities between members of the same serogroup (Mad 1 resembles B8152/66 and Ne15 is similar to M1-8 *arg his*, P22 has its own characteristics, but seems more like the former pair than the latter) However, many more strain/serogroup representatives would have to be studied before this possible correlate can be considered further

The presence of cell wall extensions and granular material around cells is probably unrelated to competence

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HYDROLYSIS OF CASEIN BY THREE EXTRACELLULAR PROTEOLYTIC ENZYMES FROM *STAPHYLOCOCCUS AUREUS*, STRAIN V8

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Three extracellular proteolytic enzymes are produced by *Staphylococcus aureus* V8 In order to develop a method to determine the activities of the individual enzymes in culture supernatants, the kinetics of the hydrolysis of casein, catalysed by purified enzymes were studied Using 1 per cent casein (pH 7 4) as a substrate, zero-order reactions were obtained for all three enzymes The K_m values were 0 59 per cent, 0 19 per cent and 0 29 per cent for protease I, II and III, respectively Using previously established data on the sensitivity of the different proteases to EDTA and cysteine, a method for the determination of the activity of each enzyme in mixed samples was developed The hydrolysis of casein in agar and agarose gels was also studied to permit interpretation of different patterns of proteolysis around colonies of staphylococci growing on casein agar media Different types of zones were obtained in the casein agar gels with the enzymes, indicating different substrate specificities Under certain conditions a specific pattern of proteolysis could be attributed to the activity of one of the proteases or a mixture of these The detection limits in casein agarose gels were approximately 25 ng/ml for protease I, 350 ng/ml for protease II, and 500 ng/ml for protease III with a test volume of 0 05 ml

Three different extracellular proteolytic enzymes produced by *Staphylococcus aureus*, strain V8 (1) have recently been purified and characterized (2, 3) All three enzymes hydrolyse casein at pH 7 5 although they have different pH optima No specific substrates have yet been found which would allow the individual proteases to be determined in mixtures such as culture filtrates Although the enzymes could be separated by gel chromatography, the total activity of the different proteases in culture supernatants could not be estimated by this method since variable inactivation occurred during the separation The present study was undertaken to compare the hydrolysis of casein by the three proteases and to find a method by which each protease could be determined separately in a mixture of all three proteases The possibility of distinguishing between different staphylococcal proteolytic enzymes by means of zymogram techniques in casein agar gels was also investigated

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MATERIALS AND METHODS

Proteases Purified proteases I, II and III from *Staphylococcus aureus*, strain V8 were used The enzymes were purified as described earlier (2, 3)

Protease assay Standard procedures were as described earlier (4) The substrate was 1 per cent casein (Nutritional Biochem Corp, Cleveland,

Ohio, USA) in 0 05 M phosphate buffer, pH 7 4, containing 1 mM Ca^{2+} and 1 mM cysteine One unit of enzyme activity was defined as the amount causing an increase of 1 0 per 30 min in the absorbance at 280 nm of perchloric acid soluble digestion products A blank was prepared for each sample tested

Casein containing agar or agarose gels were prepared by mixing 1 vol of a 2 per cent casein solution pH 7 4, containing 1 mM Ca^{++} with 1 vol of 3 per cent melted agar (Difco) or agarose (L Industrie Biologique Francaise, Gennevilliers, France) The mixture (45 50° C) was poured onto glass plates (10 × 20 cm) on a leveling table to give a 1 5 mm thick layer Wells of 6 mm in diameter were cut in the gel and the plates were stored in a moist chamber at 4° C until they were used Samples of 50 µl were placed in the wells and the gels were incubated in a moist chamber at 37° C Standard incubation time was 24 h

RESULTS

Kinetics of casein hydrolysis The rates of hydrolysis of casein were determined as a function of substrate concentration The substrate was diluted in 0 05 M phosphate buffer, pH 7 4 and the enzyme concentrations were held at 0 348 units, 0 50 units and 0 540 units /ml for proteases I, II and III, respectively The action of all three enzymes appeared to follow Michaelis Menten kinetics as shown by the linear curves obtained when the reciprocal of the rates of hydrolysis were plotted against the reciprocal of the substrate concentration according to *Lineweaver & Burk* (10) (Fig 1) The K_m and V_{max} values obtained are given in Table 1 Using 1 per cent casein (pH 7 4) as the substrate the initial rate of hydrolysis was of zero-order with respect to substrate giving a linear increase in absorbance up to 0 2 for protease I, up to 0 9 for protease II and up to 0 8 for protease III (Fig 2)

Determination of the different proteases in mixed samples It had earlier been suggested that the activity of each protease might be determined in mixed samples by the treatment of the sample with specific inhibitors for each enzyme (1) Protease III was completely inactivated by EDTA while protease I and II were unaffected by this agent (2, 3) Moreover, protease II was only active in the

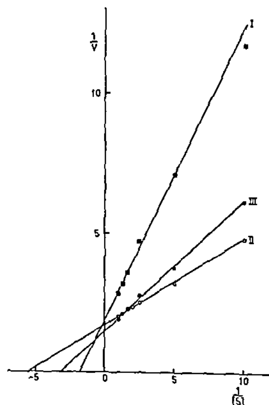


Fig 1 The rate of hydrolysis of casein catalysed by protease I, II and III as a function of substrate concentration, plotted according to *Lineweaver & Burk (10)* Velocities are given as A_{280} per 30 min, and the substrate concentrations as per cent.

presence of reducing agents such as cysteine, while protease I and III were equally active in the absence or presence of this agent Thus the activity of each enzyme in a mixture containing all three enzymes might be determined by the following procedure First, the total proteolytic activity is determined by the standard method (i e in the presence of Ca^{++} and cysteine) (value a) After treatment of the sample by EDTA (5 mM for 30 min at 20° C) the activity is again determined in the presence (value b) and in the absence of cysteine (value c) The activity of each enzyme could then be calculated as follows a minus b = *protease III*, b minus c = protease II and c = protease I

In order to test the procedure, equal volumes of purified proteases containing known

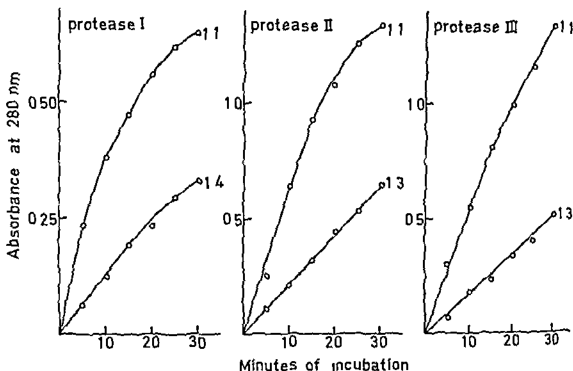


Fig 2 Hydrolysis of case n by protease I II and III as a function of time The enzyme act v ty was determ ned under standard conditions with different times of incubation Each enzyme was tested in two dilut ons as ind cated in the figure

TABLE 1 *K_m and V_{max} Values Obtained for the Hydrolysis of Casein by Protease I II and III*

Enzyme	Km	V_{max}
protease I	0 59 %	0 479 ΔA_{280} per 30 m n per μg*
protease II	0 19 %	1 770 ΔA_{280} per 30 m n per μg*
protease III	0 29 %	0 166 ΔA_{280} per 30 m n per μg*

* Based on the values of specific activity reported earl er (2 3)

amounts of enzyme activity were mixed and the mixtures were analysed as described The results are given in Table 2 The proteolytic activity of the mixtures agreed well with the theoretical values Also the activity of the individual proteases calculated from the val ues obtained by testing the mixture as indi cated in Table 2 was in good agreement with the theoretical values

Hydrolysis of casein in agar gel Different types of zones were obt ed by the different proteases (Fig 3) In ag r a distinct white precipitate was formed by protease I while proteases II and III gave very weak zones of precipitation The precipitation of the casein is partly due to degradation of K casein to insoluble para K casein (15) Hydrolysis of the K casein also results in the loss of its micelle stabilizing property causing precipita tion of whole casein complex (15) The latter reaction is the principal one leading to the formation of the precipitates seen in casein agar plates (11, 13) In agarose gels all three enzymes gave distinct zones of precipitation which differed, however in intensity and in sharpness of their margins The zones of pro-

TABLE 2 *Determination of En yme Activity of Protease I, II and III in Mixed Samples*

Assay conditions	Proteolytic activity (U/ml)				
	Protease I	Protease II	Protease III	Mixture of equal volumes of I, II and III	Theoretical activity of the mixture
a standard	1 34	2 50	2 14	2 00	2 02
b standard after EDTA treatment	1 34	2 50	0 10	1 32	1 31
c without cysteine after EDTA treatment	1 34	0 10	0 15	0 50	0 48

a b – protease III
b–c = protease II
c – protease I

Protease	Proteolytic activity (U/ml)	
	Determined in the mixture	Theoretical
I	0 50	0 48
II	0 82	0 83
III	0 68	0 71

tease I were larger in agarose than in agar gels *After precipitation of the undigested* casein by flooding the gel with 10 per cent $HClO_4$ clear zones surrounded by rings of precipitation were seen around the wells containing proteases II and III The fact that no clear area was formed by protease I indicates that this protease does not degrade the casein as extensively as proteases II and III

The sensitivity of this zymogram technique is illustrated in Fig 4 The proteolytic activity was estimated on the basis of the white precipitation zones Based on the values of specific activity of the purified enzymes (2 3), *it was calculated that protease* I could be detected at a concentration of 26 ng/ml protease II at 350 ng/ml and protease III at 500 ng/ml This means that as little as 1 3 ng of protease I in an 0 05 ml aliquot could be detected By *increasing the incubation time*, even smaller amounts of the enzymes could be detected

DISCUSSION

Different K_m values were obtained for the three proteases, indicating that they have different specificities *Drapeau et al* (6) recently reported on the purification and characterization of an extracellular protease from *Staphylococcus aureus*, strain V8 The bacterial strain was received from our laboratory and the protease studied corresponds to our protease I The same linear increase in A_{80} up to 0 2 was reported, using 1 per cent casein as the substrate Taking into consideration the limited linearity of the test of each enzyme, it was possible to determine the activity of the individual proteases in mixed *samples by specific inhibition of each activity* The accuracy of the method was about 0 05 units per ml, making it possible to follow the production of any of the proteases and to study the regulation of their formation The high K_m *value of protease I and the limited* linearity of the test compared to protease II and III, may be explained by the very narrow specificity of protease I, hydrolysing only glutamoyl bonds (9) Studies of the substrate specificity of *protease II and III* are in progress in our laboratory Preliminary experiments revealed that protease II cleaves peptide *bonds* at the amino end of leucine, isoleucine, valine, alanine and phenylalanine

Also the *different types of zones of* proteo-

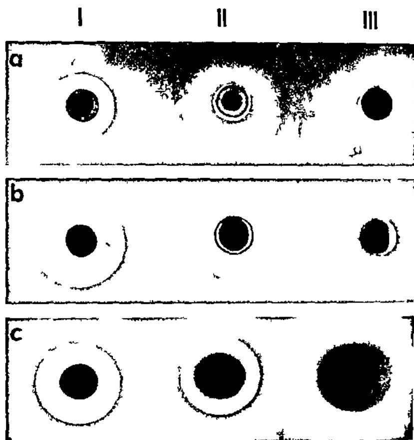


Fig 3 Activity of protease I II and III against casein in agar (a) and agarose (b) gels Enzyme concentrations were the same as in Fig 4 After incubation at 37° C for 24 h the agarose gel was flooded with 10 per cent $HClO_4$ (c)

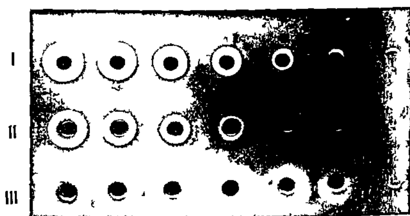


Fig 4 Sensitivity of the casein agarose method Serial 2 fold dilutions of the proteases were prepared in distilled water and 50 µl aliquots placed in the wells in a casein agarose gel The highest concentrations of proteases I was 0 125 units/ml (specific activity 300 units/mg) 4 0 units/ml of protease II (specific activity 1500 units/mg) and 1 75 units/ml of protease III (specific activity 120 units/ml) The gel was incubated at 3[illegible] C for 24 h The proteolytic activity was estimated on the basis of the white precipitation zones. (The diffuse large zones seen with protease II are probably the result of diffusion of the samples between the agarose gel and the glass plate)

lysis obtained on casein agar indicated that the three proteases have different substrate specificities The inability of protease I to further degrade the precipitate formed by partial hydrolysis of the K-casein is in good agreement with the narrow substrate specificity of this enzyme (9) Judged from the type of zones on casein-agar gels, protease III exhibits a broader specificity than both proteases I and II

The greater sensitivity of the casein precipitating reaction compared to the Kunitz method has been reported by *Fossum* (8) Trypsin at a concentration of about 200 ng/ml was detected in 0 025 ml aliquots, corresponding to a total amount of only 5 ng About the same small amounts of the staphylococcal proteases gave visible zones of proteolysis on casein agarose gels making the method suitable for detection of the different staphylococcal proteases after electrophoresis or isoelectric focusing of crude mixtures (i e culture supernatants) in agar or polyacrylamide gels By covering the electrophoresis or electrofucusing gels with a layer of casein-agarose, the separated enzymes could be visualized and immediately identified, thanks to their characteristic patterns of proteolysis (unpublished results)

Since most staphylococci seem to be proteolytic (7), attempts have been made to differentiate strains of staphylococci by means of serological typing of their proteins (5, 14) The differentiation of coagulase-positive staphylococci by the patterns of precipitation on caseinate agar medium has been reported by *Martley et al* (12) Five groups could be defined (A-E) *Staphylococcus aureus*, strain V8 belongs to group A giving a narrow ring of precipitation surrounding a broad clear area The precipitation is the result of protease I activity and the transparent zone is the result of protease III degradation of the precipitate formed by protease I Type D zones, according to *Martley et al*, are characterized only by precipitation and should consequently be formed by strains producing only protease I, or a similar protease The other types of zones (B and C) may be caused by different combination of protease I, II and III, or other enzymes Group E strains were nonproteolytic

Since completely different patterns of proteolysis can be obtained by altering the proportion of two or more proteases, the typing of staphylococci according to *Martley et al* may not be reliable until the proteases responsible for the different types of zones have been characterized So far, the three enzymes discussed in the present paper are the only staphylococcal proteases which have been purified and characterized (2, 3, 6) It is possible that studies of only a few strains of staphylococci belonging to each group according to *Martley et al* could reveal most types of proteases produced by staphylococci

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THE ROLE OF CALCIUM FOR STABILITY AND ACTIVITY OF AN EXTRACELLULAR PROTEOLYTIC ENZYME FROM *STAPHYLOCOCCUS AUREUS*

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The inactivation by EDTA of an extracellular protease, produced by *Staphylococcus aureus*, strain V8, was studied by the use of radioactive enzyme, gel chromatography and quantitative immunological methods It was shown that the removal of Ca^{++} from the enzyme by treatment with EDTA (5 mM) at 4° C resulted in irreversible loss of its activity on casein This inactivation was not accompanied by any measurable change in the molecular weight or immunoprecipitating properties of the protease If treatment with EDTA were performed at 37° C, the loss of activity on casein would be followed by a subsequent loss of the immunoprecipitating properties of the enzyme, which was shown to be due to degradation of the enzyme The degradation started after the loss of detectable enzyme activity Thus it may be suggested that the removal of Ca^{++} from the enzyme resulted in an altered tertiary structure of the protease in which the active site was hidden so that it could not reach the large substrate molecules i e casein and that, though inactive against casein, the calcium free enzyme was capable of degrading itself to peptides with molecular weights in the range 4 000 7,000

Three extracellular proteolytic enzymes are produced by *Staphylococcus aureus*, strain V8 (2) One of these, protease III, was irreversibly inactivated by EDTA (3) It was also observed that Ca^{++} ions prevented rapid inactivation of the enzyme during the purification procedures The role of calcium for the maintenance of stability of a number of different proteases, including trypsin (13, 14), neutral proteases from various *Bacillus* species (11, 12, 15, 19), *Bacillus subtilis* alkaline protease (16) *Pseudomonas aeruginosa* protease (21), *Streptomyces griseus* neutral protease (24), a neutral protease from *Cytophaga* sp (9) and also proteases from various micrococci (7, 10) has been reported In the case of some proteases it has been shown that the presence of Ca^{++} prevents autodigestion of the enzymes (7, 11) Moreover, several of the proteases stabilized by Ca^{2+} needed zinc for their enzyme activity However, zinc seemed to be of no importance in proteases from micrococci (10) or in *Staphylococcus aureus* protease III (3) In the present paper, the mechanism of inactivation of protease III by EDTA was studied to find out the role of Ca^{++} ions in this enzyme

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MATERIALS AND METHODS

Protease Purified protease III from *Staphylococcus aureus* strain V8, was prepared as described earlier (3) Radioactive protease was prepared by

adding a mixture of 15 ^{14}C L amino acids (NEC 445, New England Nuclear Corp) to the culture medium giving an activity of 0 5 $\mu Ci/ml$ Protease III was purified by gel chromatography as previously described (3) The purified enzyme was free of detectable protease I and II and gave only one line of precipitation on immunoelectrophoresis The purified enzyme was kept at 4° C in the presence of 1 0 mM Ca^{2+}

Gel chromatography Sephadex G 75 (Pharmacia Uppsala Sweden) was packed by gravity in a 140×2 5 cm vertical column and equilibrated with 0 05 M Tris/HCl buffer pH 7 2 containing 1 0 mM Ca^{2+} The flow rate during the equilibration and the chromatography was 12 ml/h All experiments were run at 4° C Six ml samples were collected A Sephadex G 15 column (100×2 5 cm) equilibrated with 0 25 M acetic acid was used for the chromatography of autodigestion products Elution rate and sample collection were as for the Sephadex G 75 column Samples (0 1 ml) of the chromatographic eluent were counted in a liquid scintillation counter (Mark I, Nuclear Chicago Corp) using 10 ml of a dioxan based scintillation fluid For estimation of molecular weights the following markers were used Blue dextran 2000 (Pharmacia), ovalbumin (Sigma), cytochrome c (horse heart, Sigma) and ^{14}C L valine (New England Nuclear Corp) Molecular weights were calculated according to *Andrews* (1)

Electro immuno assay Electrophoresis in antibody containing agarose gels was performed according to *Laurell* (18) Agarose, 1 per cent w/v (Miles Seravac Berks, England) containing 2 µl/ml of polyvalent rabbit antiserum against *Staphylococcus aureus*, strain V8 culture filtrates was used The antiserum was prepared as described earlier (4) The electrophoresis experiments were run for 15 h at 4 V/cm in 0 07 M barbitone buffer pH 8 6 containing 2 mM calcium lactate Samples (10 µl) were placed in wells of 4 mm diameter at the cathodal end of the 10×10×0 15 cm gel After electrophoresis the gels were dried (18) and stained with Coomassie brilliant blue (8)

Enzyme assay Proteolytic activity was determined by the method of Kunitz using casein as the substrate (20) and modified as described earlier (5) The assay was carried out at 37° C in the presence of Ca^{2+} at a concentration of 1 0 mM For specific assay of protease III in samples containing the other staphylococcal proteases I and II, proteolytic activity was determined before and after treatment of the sample by EDTA at a concentration of 5 mM for 30 min at 20° C (6) For the detection of small amounts of active protease the casein agar plate method was used (6)

RESULTS

The rates of inactivation of protease III by EDTA at 4° C and 37° C have been reported earlier (3) Enzyme activity was completely lost within 2 min at 37° C and in about 5 min at 4° C in the presence of EDTA at a concentration of 5 mM This experiment was repeated in the following way Samples containing appropriate amounts of protease III were diluted in EDTA in 0 1 M phosphate buffer, pH 7 0, at 4° C and 37° C Final concentration of EDTA was 5 mM Aliquots were removed at intervals and immediately diluted 1 4 in cold 10 mM CaCl Each sample was assayed for proteolytic activity and analysed by electro immuno assay (Fig 1) As seen in the figure, enzyme activity disappeared (as reported earlier) in less than 2 min at 37° C and in about 6 min at 4° C In addition, the immunoprecipitating property of the protease was lost at 37° C, but at a slower rate than the enzymatic activity At 4° C, any decrease in the height of the precipitation peak was not seen in 15 min though the enzyme was completely inactivated in about 5 min Analysis of 2 fold serial dilutions of protease III under identical conditions showed that the height of the precipitation peak was proportional to the amount of antigen as described by *Laurell* (17) The present results indicate that two distinct events occurred following treatment of protease III with EDTA First the enzymatic activity against casein was lost by the removal of Ca^{2+} and then the immunoprecipitating properties disappeared, suggesting degradation of the enzyme Both reactions were irreversible but could be stopped or slowed down at any time by the addition of Ca^{2+} (first reaction) and by lowering the temperature (second reaction)

To investigate whether loss of immunoprecipitating properties might be due to degradation of protease III, radioactive enzyme was treated with EDTA (5 mM) for 20 min at 37° C and run through a Sephadex G 75 column (Fig 2) About 70 per cent of the radioactivity applied on the column was

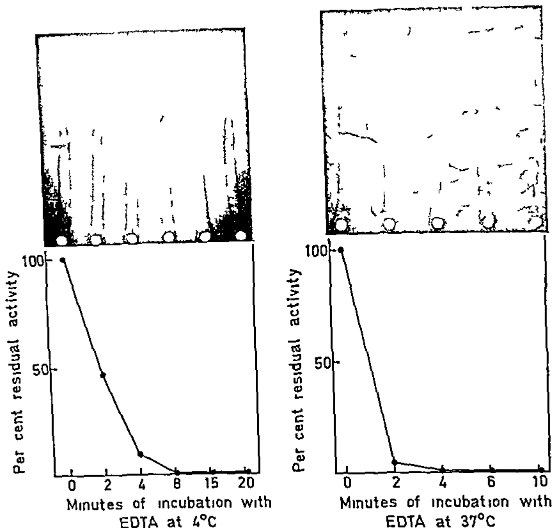


Fig 1 Inact at on of protease III by EDTA at 4° C and 37° C The proteolyt c activity and the immunoprecip tat on reactions are represented on the same t me ax s

eluted in one peak just after cytochrome c and before ^{14}C valine No protease activity could be detected in the elution fluid by means of the casein agar plate method The molecular weights of these labelled compounds were in the range of 4 000–7,000 Thus based on the earlier reported value of the molecular weight of protease III, 28 000 (3) the enzyme appeared to be split in at least three different places These low molecular weight moieties gave no precipitation reaction with a polyvalent antiserum but absorbed protease III specific antibodies (Fig 3), providing evidence that they were degradation products of protease III Gel chromatography on Sephadex G 75 of radioactive protease III treated with EDTA at 4° C did not reveal any degradation products and the inactive enzyme was eluted by the same volume as active protease

The purity of the protease III preparation used judged from the immunoelectrophoresis (Fig 1) and the fact that no protease activity was detected in the elution fluid by the very sensitive casein agar method (6) and that no high molecular weight component was de

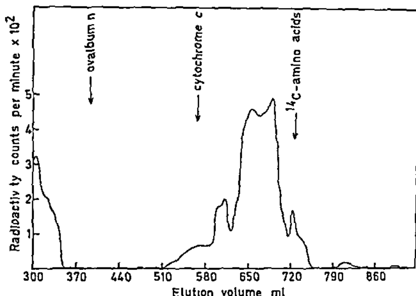


Fig 2 Gel chromatography on Sephadex G 75 of radioactive protease treated with EDTA for 20 min at 37° C The arrows indicate the elution volumes of the marker molecules ovalbumin cytochrome c and ^{14}C L valine

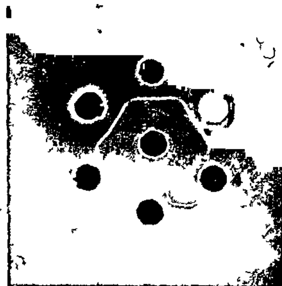


Fig 3 Immunodiffusion in agarose gel In the central well was placed fully *active protease III* In well 1 and 2 polyvalent antiserum in well 3 antiserum diluted 1 2 in distilled water and in well 4 antiserum diluted 1 2 in protease III which had been inactivated by EDTA (5 mM at 37° C)

tected after degradation of protease III supported the suggestion that the enzyme was degraded through autodigestion

It had also been observed that protease *III* was irreversibly inactivated also in the presence of Ca^{2+} ions although at a lower rate than after treatment by EDTA (3) The following experiments were performed to elucidate the mechanism of this inactivation Radioactive enzyme (1125 cpm/unit) was incubated for 18 h at 37° C in the presence of Ca at a concentration of 10 mM The enzymic activity decreased by 70 per cent After chromatography of the partly inactivated protease on a Sephadex G 75 column at 4° C two peaks of radioactivity were eluted (Fig 4) The first peak contained more than 90 per cent of the proteolytic activity applied to the column whereas the second peak exhibited no proteolytic activity The specific activity of the recovered protease was 1077 cpm/unit indicating that *it was completely* separated from inactive enzyme The molecular weight of the active enzyme was about 30 000 which is in good agreement with the *previously reported* value (28 000) for protease III (3) The second peak was eluted by a larger volume than the products of the EDTA inactivated protease The difference in size was also confirmed by the fact that most of the split products from protease in-

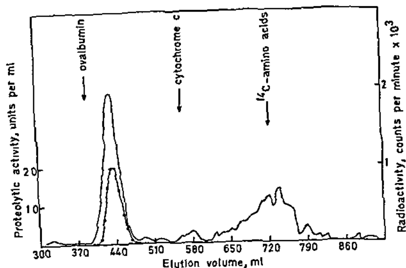


Fig 4 Gel chromatography on Sephadex G 75 of radioactive protease III incubated for 18 h at 37° C in the presence of Ca^{2+} at a concentration of 1 0 mM Proteolytic activity, ●——●, radioactivity, —— The arrows indicate the elution volumes of the marker molecules ovalbumin, cytochrome c and ^{14}C L valine

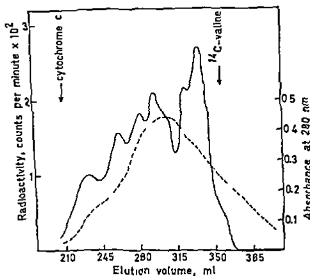


Fig 5 Gel chromatography on Sephadex G 15 of the degradation products of radioactive protease III incubated in the presence of Ca^{2+} at 37° C The void volume (indicated by the arrow) was determined by the use of cytochrome c radioactivity ——, absorbance at 280 nm – – –

activated in the presence of Ca^{2+} were included in Sephadex G 15 which excludes molecules larger than 1500 (Fig 5) The low molecular weight products of the second peak could not be identified as degradation products of protease III by absorption of antibodies However, the fact that the inactivation was not accompanied by any change in the specific activity of the remaining protease was in support of the assumption that the inactivation was due to degradation of the enzyme.

DISCUSSION

Though it has not been conclusively shown that protease III contains calcium, there is much evidence in support of this assumption (3) and it is suggested that calcium is removed by the addition of EDTA The present results showed that two distinct events occurred following treatment of protease III with EDTA First the catalytic activity toward casein was lost This inactivation was irreversible and was not accompanied by any change in the molecular weight or antigenic properties of the enzyme It is suggested that the removal of calcium resulted in a conformational change of the protease hiding the active site so that it could not attack the high molecular weight substrate i e casein The second phase of the inactivation was degradation, leading to the disappearance of the immunoprecipitating property of the enzyme and a complete loss of the catalytic capacity The antigenic sites of the protease were still present on the degradation products as judged from their ability to absorb protease III specific antibodies Though not conclusively proved, the present data supports the suggestion that the degradation of protease III could be due to autodigestion Final proof will not be obtained until the peptide bonds split during degradation can be compared with the substrate specificity of the enzyme So far, only preliminary data on the substrate specificity are available, indicating that protease III attacks peptide bonds involving the amino groups of alanine, valine leucine isoleucine and phenylalanine (to be published)

The role of calcium for the maintenance of an active tertiary structure of a snake venom protease has been reported (25) The removal of Ca^{2+} from this enzyme resulted in an altered conformation rendering the enzyme irreversibly inactive against casein No autodigestion of the enzyme was observed However in the case of several bacterial proteases it has been shown that the role of calcium was to prevent autodigestion (10 11 19, 22, 26) The significance of calcium for the catalytic activity is generally unknown In the case of neutral proteases from various Bacillus species calcium was essential for the stability of the enzymes whereas zinc was essential for the enzymic activity (11, 19) Simultaneous removal of calcium and zinc from thermolysin yielded an inactive apoenzyme which did not autolyse (11), indicating that an intact active site was essential for autodigestion Accordingly, since autodigestion of protease III occurred after the removal of calcium, it was suggested that calcium was not in the active site of this enzyme

Some bacterial proteases are produced only in media containing calcium (23 26), a feature common to protease III (2) In the case of a protease from *Sarcina* (coccus P) it has been reported that the only role of calcium in production was to stabilize the enzyme (7) A similar mechanism is suggested to be involved in protease III

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THE FORMATION OF A CALCIUM-DEPENDENT EXTRACELLULAR PROTEOLYTIC ENZYME FROM *STAPHYLOCOCCUS AUREUS*

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The role of calcium in the production of an extracellular proteolytic enzyme (protease III) from *Staphylococcus aureus*, strain V8 has been studied No protease III was found in cultures grown in a casein hydrolysate medium containing EDTA at a concentration of 0.1 mM Addition of Ca^{2+} during growth in this medium resulted in an accumulation of protease III in the culture The appearance of protease was the result of *de novo protein* synthesis No inactive protease III could be detected in cultures grown in the presence of EDTA by electrophoresis in antibody containing agarose gels However, protease III specific antibodies could be absorbed or blocked by supernatant fluids from cultures grown in the presence of EDTA, *indicating that degradation products of the enzyme possessing antigenic determinants* were present It was concluded that calcium was neither necessary for synthesis nor for excretion of protease III but was required for the stability of the enzyme

One of the extracellular proteases produced by *Staphylococcus aureus*, strain V 8 was only found in cultures grown in the presence of Ca^{2+} (2), a feature common to several bacterial exo-enzymes (19) including proteases (7, 12, 18, 21) The precise role of Ca^{2+} in exoenzyme production is not known, but it has been suggested that this metal is involved in the excretion mechanism (19) In the case of extracellular proteases it has been suggested that the role of Ca^{2+} is to prevent inactivation of the enzymes through autodigestion (11)

The role of calcium ions for the activity and stability of the calciumdependent protease (protease III) has been studied (5) Their presence was necessary for the maintainance of full activity of the enzyme Removal by EDTA resulted in an irreversible loss of enzyme activity against casein Furthermore, the calcium-free protease was rapidly degraded to small peptides These findings suggested that calcium might function by stabilizing the enzyme during production The present study was undertaken to test this hypothesis

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MATERIALS AND METHODS

Bacterial strain and cultivation conditions Staphylococcus aureus, strain V8 (13) was cultivated in 1 l baffled Erlenmeyer flasks on a rotary shaker (50 mm displacement, 120 rev/min) at 37° C The culture volume was 100 or 50 ml Precultures and inoculum procedures have been described earlier (13)

Culture medium The basal medium was the previously described CC medium (2) based on the CCY medium of *Gladstone and van Heyningen* (9) The CC medium had the following composition casein hydrolysate (*Oxoid*), 40 g Na-β-glycerophosphate, 20 g, Na-lactate (50 per cent),

10 ml, Na_2HPO_4 $2H_2O$, 1 g, KH_2PO_4, 0 4 g,

of 1000 ml The vitamins and the divalent metal ions were sterilized separately at 100 times the required concentration The calcium content of the medium was determined as 0 02 mM by atomic absorption (kindly performed by Dr *A Bocallius*, Research Institute of Swedish National Defence) If necessary, a sterile solution of 1 M $CaCl_2$ was added to the medium to give a final concentration of 1 0 mM To reduce the concentration of Ca^{++} in the medium, EDTA was added to a final concentration of 0 1 mM

Determination of bacterial growth Bacterial growth was determined by dry weight measurements and by the incorporation of a radioactive amino acid ^{14}C-L-leucine (New England Nuclear Corp , Boston, Mass , U S A) was added to growing cultures at a final concentration of 0 04 μg/ml (specific activity, 655 μCi/mole) Samples of 0 2 ml were removed at intervals and immediately mixed with 5 ml of cold 10 per cent (w/v) trichloracetic acid The precipitates were collected on a membrane filter (Millipore Corp , 0 45 nm pore size) and washed repeatedly with trichloracetic acid The filters were counted in a liquid scintillation counter (Nuclear Chicago, Mark I) using 5 ml of a toluene based scintillation fluid

Enzyme assay Protease activity was determined by the method of *Kunitz* (15) modified as described earlier (1), using casein as the substrate For determination of protease III activity in samples containing the other extracellular proteases, protease I and II, the samples were tested before and after treatment with EDTA at a concentration of 5 0 mM for 30 min at 20° C (4)

Immunological methods Electrophoresis in antibody containing agarose gels according to *Laurell* (16) was carried out as previously described (5), using polyvalent rabbit antiserum to *Staphylococcus aureus*, strain V8 culture supernatant prepared as described earlier (3)

Immunodiffusion according to Ouchterlony was performed in 1 per cent agarose gels, 1 5 mm thick, with wells (2 mm diameter) cut with a gel punch (LKB Produkter, Stockholm, Sweden, model 6808A)

Chemicals Casein was purchased from Nutritional Biochem Corp , Cleveland, Ohio, U S A , agarose from Miles Seravac, Berks, England, and rifampicin from CIBA Geigy AG, Basel, Switzerland All other chemicals were of analytic grade, purchased from AB KEBO, Stockholm, Sweden

RESULTS

It was reported that no protease III activity was found in supernatant fluids of cultures grown in the CC-medium unless extra Ca^{++} was added (2) However, in subsequent experiments small amounts of protease III were occasionally obtained in this medium This was due to variations in the calcium content of the casein hydrolysate which is the main source of this metal in the medium The

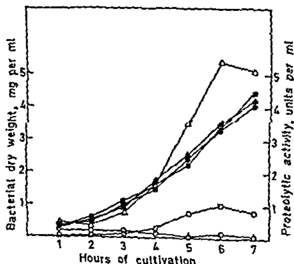


Fig 1 Bacterial growth (solid symbols) and protease III production (open symbols) in CC medium, ■ and □, CC-medium with 0 1 mM EDTA, ● and ○ and CC medium with 1 0 mM Ca^{++}, ▲ and △

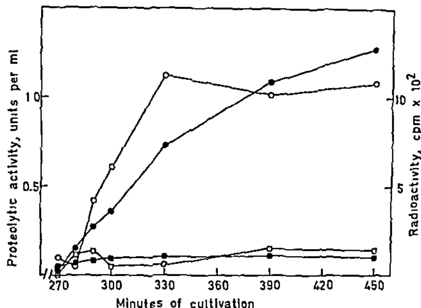


Fig 2 Production of protease III after addition of Ca^{2+} to cultures grown for 4 5 h in CC medium containing 0 1 mM EDTA and the effect of rifampicin on this production O— O proteolytic activity in culture to which Ca^{2+} and ^{14}C L leucine were added, ●—●, incorporation of ^{14}C L leucine in the same culture, □—□, proteolytic activity in culture to which Ca^{2+}, ^{14}C L leucine and rifampicin were added, ■—■, incorporation of ^{14}C L leucine in the same culture

batch of casein hydrolysate used in this study gave the CC medium a final calcium concentration of 0 02 mM Decreasing the concentration of Ca^{2+} ions in the medium by the addition of EDTA at a final concentration of 0 1 mM resulted in total inhibition of protease III production (Fig 1) Bacterial growth was not affected by EDTA at this concentration In Fig 1 the bacterial growth and protease III production in the basal CC medium and in CC medium with extra Ca^{2+} (final concentration, 1 mM) are also shown The addition of extra Ca^{2+} to the CC-medium provided a five-fold increase in the production of protease III without affecting the bacterial growth

In order to investigate whether bacteria in the presence of EDTA accumulated intracellular enzyme or excreted a zymogen, the following experiments were carried out The bacteria were cultivated in CC medium containing EDTA at a concentration of 0 1 mM After growth for 4 5 h, ^{14}C-leucine was added and the culture immediately divided into two equal portions To one portion, Ca^{2+} was added to a final concentration of 1 0 mM and to the other, Ca^{2+} to the same concentration together with rifampicin (10 μg/ml) Both cultures were incubated at 37° C on the rotary shaker As seen in Fig 2, protease III activity appeared in the culture immediately after the addition of Ca^{2+} The accumulation of protease continued for about 60 min upon which it remained at the same level for 2 h while the incorporation of radioactivity continued throughout the experiment However, at the time when protease production stopped there was a decrease in the rate of incorporation of radioactivity, indicating that some nutritional factor in the medium was exhausted If Ca^{2+} were added later than after 5 5 h of cultivation no protease III would be obtained, indicating that the enzyme was not synthesized after this time and that no protease III had accumulated intracellularly during the early phase of growth in the absence of Ca^{2+} The fact that the appearance of protease III after the addition of Ca^{2+} was totally inhibited by rifampicin (Fig 2) confirmed the suggestion that production was not

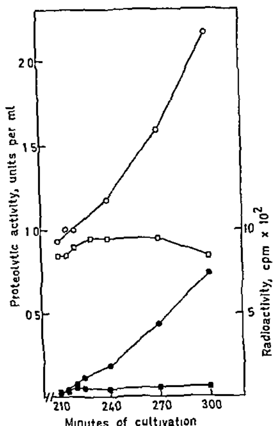


Fig 3 Effect of rifampicin on production of protease III in CC medium containing Ca^{2+} at a concentration of 1 0 mM Rifampicin 10 µg/ml and ^{14}C L leucine were added after 3 5 h of cultivation To one control only ^{14}C L leucine was added □—□ proteolytic activity in the culture to which rifampicin was added, ■—■, incorporation of ^{14}C L leucine in the same culture ○—○, proteolytic activity in the control ●—●, incorporation of ^{14}C L-leucine in the control

merely due to release of preformed enzyme, but resulted from *de novo* protein synthesis As a control rifampicin was added to cultures grown in the presence of Ca^{2+} (1 0 mM) (Fig 3) No significant protease III production was obtained in the presence of rifampicin, indicating that bacteria producing active protease III did not accumulate cell bound enzyme which could have been released if Ca^{2+} was added

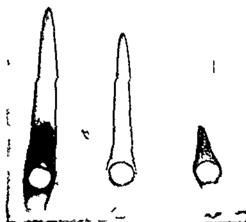
It may be suggested on the basis of these results and the earlier finding that the presence of calcium prevented inactivation and autodigestion of protease III, that equal amounts of the enzyme were synthesized and excreted regardless of the calcium content in the culture medium It would then be possible to find an inactive protease or degradation products in calcium-deficient cultures As reported earlier (5), the inactive calcium free form of the enzyme was rapidly degraded at 37° C and lost its immunoprecipitating properties However, the degradation products were still able to absorb protease III specific antibodies

Electro immuno assay of samples from cultures grown for 6 h in CC medium and in CC medium with 1 0 mM Ca^{2+} gave two peaks of precipitation (Fig 4 a and 4 b) As seen in the figures, dilution of the samples 1 2 decreased the height of the peaks to about one half of the original, indicating that the heights of the peaks were proportional to the amount of antigen, as described by *Laurell* (16) The two different peaks were identified as protease I and protease III by comparison with the precipitins obtained with purified enzymes (Fig 5) As seen in Fig 4 the peak corresponding to protease III was considerably higher with the sample from the calcium enriched medium than with that from the ordinary CC medium, indicating that the lower protease III activity in the calcium deficient medium could not be accounted for by the presence in the culture supernatant of enzymically inactive protein with immunoprecipitating properties Analysis of samples from cultures grown in the presence of EDTA gave only one peak of precipitation which was of the protease I type (Fig 4 c)

In order to detect degradation products of protease III in culture supernatants from cultures grown in the presence of EDTA, the ability of these supernatants to absorb protease III specific antibodies was analysed by immunodiffusion in agarose gels

Polyvalent antiserum was diluted 1 2 in distilled water and in culture supernatant fluid concentrated ten times The diluted sera were allowed to diffuse against purified protease III (Fig 6) As a control serum diluted in a similarly concentrated culture super-

Fig 4 Electrophoresis in antibody containing agarose gels of supernatant fluids from cultures grown in CC medium with 1 mM Ca^{2+} (a) CC medium (b) CC medium with 0.1 mM EDTA (c) Culture supernatants had been concentrated 5 times and dialysed against 0.1 mM $CaCl_2$ at 4° C Equivalent amounts per mg bacterial dry weight were applied Electrophoresis was performed at 3 V/cm for 18 h in 0.07 M barbitone buffer pH 8.6 Each sample was applied in two fold dilutions



natant from a culture grown in presence of extra Ca^{2+} was used Before mixing with the serum this supernatant was treated with EDTA (5 mM for 20 min at 37° C) in order to inactivate protease III and permit autodigestion of the enzyme As seen in Fig 6 significant amounts of protease III specific antibodies were absorbed or blocked by the supernatant from the culture grown in the presence of EDTA About the same blocking effect was obtained using the EDTA inactivated supernatant from the culture grown in the presence of Ca The present results support the hypothesis that protease III is synthesized and excreted by bacteria grown

Fig 5 Electrophoresis in antibody containing agarose gel of purified protease I and III From *left to right* mixture of the enzymes protease III protease I

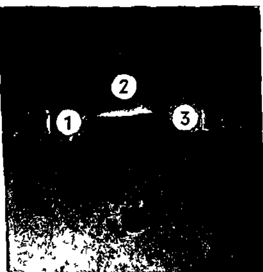


Fig 6 Immunodiffusion according to Ouchterlony The central well contained purified protease III well 1 polyvalent antiserum diluted 1 2 in super natant fluid from a culture grown in presence of 1 mM Ca^{2+} treated with EDTA, well 2 polyvalent antiserum diluted 1 2 in distilled water, and well 3, polyvalent antiserum diluted 1 2 in supernatant flu d from a culture grown in the presence of EDTA

in the presence of EDTA, but that calcium is needed for the stability of the enzyme

DISCUSSION

The main purpose of the present study was to investigate the role of Ca^{2} for the production of protease III Since no completely calciumfree medium could be obtained, EDTA was added in order to reduce the concentration of Ca^{2+}-ions in the CC medium (2×10^{-5} M) The low concentration of EDTA required for total inhibition of pro tease production and the known high affinity of EDTA for Ca compared with other metal ions present in the medium i e Mg^{+} Zn^{+} Fe^{2+} and Mn^{+} (2) is in support of the assumption that inhibition of production was due to the binding of Ca^{+} ions which were shown to have a stimulative effect on production

The experiments with rifampicin showed that the appearance of protease III after the addition of Ca^{+} to bacteria grown in the presence of EDTA was dependent on messenger RNA synthesis and consequently *de novo* protein synthesis These results and the earlier finding that only trace amounts of protease were present intracellularly (2) indicated that the enzyme was excreted im mediately after it had been synthesized and that no intracellular pool of the protease had accumulated during growth either in the presence or absence of calcium ions It is thus suggested that these ions are not required for the excretion of the enzyme A more direct indication that protease III is excreted irrespective of the calcium concentration of the medium is that degradation products of the enzyme were found in the supernatant fluid of cultures grown in the presence of EDTA Furthermore, about the same degree of ab sorption of protease III specific antibodies was obtained with equivalent amounts of culture fluid from cultures grown with EDTA and with extra Ca^{2+}, indicating that the same amount of protease was synthesized and extruded into both media, the enzyme being immediately degraded after excretion in the former case

The mechanism of excretion and binding of Ca^{2+} to protease III is not yet known However, the model for extracellular enzyme production suggested by *May* & *Elliot* (17) may be applied to the present data According to this model, enzyme synthesis occurs on ribosomes bound at special translation ex trusion sites located on the cell membrane the polypeptide chain being excreted directly as it is synthesized, to assume its three dimensional form outside the membrane Taking into consideration the instability of the calcium free inactive form of protease III and the inability to reactivate the enzyme (5), it is suggested that Ca^{2+} ions have to be present at the extrusion site for the formation of an active conformation of the enzyme

Assuming a low cysteine/cystine content of protease III, according to the proposal of *Pollock* & *Richmond* (20) for bacterial extracellular proteins, the role of calcium for the maintenance of an active and more stable tertiary structure of protease III may be to

form electrostatic bridges in place of disulphide bonds This role of calcium has been suggested in the case of σ amylase (14), an extracellular protease from a *Sarcina* strain Coccus P (6) and thermolysin (8)

In the case of the protease from the *Sarcina* strain which was only produced in media containing Ca^{2+}, *Bissell et al* (6) showed that the sole effect of these ions was to arrest degradation of the enzyme molecule after its release These authors also suggested that the enzyme might be excreted in an unfolded or partly folded form which, in contrast to protease III, did not require Ca^{2+} for its activation Activation involved autocatalysis of a zymogen (10), the active enzyme being stabilized by Ca^{2+}

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DNA BASE COMPOSITION OF COAGULASE-NEGATIVE STAPHYLOCOCCI ASSOCIATED WITH URINARY TRACT INFECTION

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DNA base composition (per cent GC) of thirteen coagulase negative staphylococci has been determined The strains originate from urinary tract infection and include members both of the genus *Micrococcus* and *Staphylococcus* in the sense laid down by the *Subcommittee on Taxonomy of Staphylococci and Micrococci* All strains were found to have a per cent GC in the range typical of staphylococci (30–40) It is concluded that this observation supports the proposal by *Baird Parker* to place organisms of this type in the genus *Staphylococcus*

Gram positive, catalase positive, coagulase negative cocci have in recent years been isolated under circumstances pointing to their possible role as pathogens in urinary tract infection

Various names have been assigned to the isolates, e g coagulase negative *Staphylococcus albus* (*Pereira* 1962), *Staphylococcus albus* (*Mitchell* 1964 *Pulverer et al* 1972) coagulase negative staphylococci (*Gallagher et al* 1965, *Mabeck* 1969), *Staphylococcus albus*, *Staphylococcus epidermidis* and *Micrococcus* with subgroups (*Corse* & *Williams* 1968) or *Staphylococcus* and *Micrococcus* with subgroups (*Roberts* 1967)

The *Subcommittee on Taxonomy of Staphylococci and Micrococci* (1965a,1965b) has recommended determination of the anaerobic production of acid from glucose to be used in the distinction between genus *Micrococcus* and *Staphylococcus* The urinary pathogenic strains differ in this character Some are identified as *Micrococcus* strains and others as *Staphylococcus* strains (*Roberts* 1967, *Corse* & *Williams* 1968, *Mortensen* 1969)

Examination of the base composition of deoxyribonucleic acid (DNA) expressed as a mole percentage of guanine and cytosine to total bases (per cent GC) of staphylococcal and micrococcal strains shows the existence of two groups one which includes typical staphylococci with a DNA base composition of 30–40 per cent GC and one including typical micrococci with 50–75 per cent GC (*Kocur et al* 1971) However, organisms both from the high (50–75) and low (30–40) per cent GC groups have been identified as members of the genus *Micrococcus* by the criterion set up by the *Subcommittee* (e g *Auletta* & *Kennedy* 1966)

The purpose of this report is to examine the per cent GC of pathogenic coagulase-

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negative bacterial strains that belong to the family *Micrococcaceae* and which, according to the test recommended by the Subcommittee, include *Staphylococcus* as well as *Micrococcus* strains

MATERIALS AND METHODS

Bacterial strains and growth of bacteria for DNA isolation Thirteen strains originally isolated by *Mabeck* (1969) were examined They were selected among the strains examined by *Mortensen* (1969) who identified five of the 13 strains as *Staphylococcus* and eight strains as *Micrococcus* The selection includes at least one representative of each of the ten biochemical patterns listed in his Table II (1969)

From each strain a single colony was isolated and inoculated in 5 ml broth After overnight incubation the culture was transferred to 500 ml Brain Heart broth (Difco) and incubated at 37° C under shaking for 24 h At this time, a sample of bacteria was taken for purity control and re identification

Lysis of the organisms and DNA preparation None of the strains were sensitive to lysozyme However spheroplast formation was in all cases found possible by addition of either penicillin (*Silvestri* & *Hill* 1965) ampicillin or methicillin to the growth medium The spheroplasts were collected by centrifugation and the DNA extracted and purified according to *Marmur* (1961) The finally purified DNA was dissolved in SSC (0 15 M NaCl 0 015 M trisodiumcitrate pH 7 0) at a concentration of approximately 100 μg DNA/ml This solution was stored at −20° C until used

Determination of mean denaturation temperature of DNA Thermal denaturation was done essentially as described by *Stenderup* & *Bak* (1968) except that the water heated cuvette holder of the spectrophotometer was replaced by an electronically controlled and heated holder The temperature increase used was 1/4° C/min The temperature correction of the thermosensor was + 0 2° C in the temperature interval in which the melting temperatures were found Calculations were done according to *Marmur* & *Doty* (1962)

Determination of buoyant density of DNA A Beckmann model E analytical ultracentrifuge equipped with monochromator, multiplexer and scanning system was used for this determination All runs were at 25 0° C 44 000 rev/min in the AnG rotor using double sector charcoal filled epon centerpieces All scannings were done after 20 h centrifugation using the medium scan speed and a wavelength of 265 nm As a reference DNA from *Micrococcus lysodeikticus* (density 1 731 g/ccm) or DNA from *E coli* B (density 1 710 g/ccm) was used Preparations of solutions and calculations were as described by *Bak et al* (1972)

TABLE 1 *Mean Denaturation Temperatures (Tm) and Buoyant Densities (BD) and Calculated % GC of DNA Isolated from Thirteen Strains of Gram positive Cocci*

Patient & strain*	Genus†	Tm° C§	Mole % GC calculated from Tm	Buoyant density§ g/ccm	Mole % GC calculated from BD
VC 6912		82 8 0 2 (5)	32 8	1 6932 0 0007 (4)	33 9
NT 278		82 7 0 3 (5)	32 6	1 6927 0 0005 (3)	33 4
UH 432	*Staphylococcus*	82 8 0 1 (4)	32 8	1 6932 0 0004 (4)	33 8
BMA 558		82 8 0 1 (5)	32 8	1 6932 0 0003 (3)	33 9
MN 6179		82 9 0 2 (3)	33 1	1 6932 0 0002 (2)	33 9
IM 6498		83 0 0 3 (3)	33 4	1 6936 0 0002 (2)	34 2
EN 6148 (phage typable)		83 2 0 3 (4)	33 8	1 6938 0 0001 (2)	34 5
SP 39		83 2 0 2 (4)	33 8	1 6938 0 0008 (4)	34 5
MH 6710 (La O)		83 3 0 2 (4)	34 0	1 6941 0 0004 (3)	34 8
MH 536 (La +)	*Micrococcus*	83 1 0 1 (4)	33 7	1 6938 0 0008 (3)	34 5
KH 693		83 2 0 2 (5)	33 8	1 6937 0 0007 (4)	34 4
LH 6495		83 3 0 2 (5)	34 0	1 6938 0 0005 (3)	34 5
KJ 6705		83 1 0 2 (4)	33 7	1 6939 0 0008 (3)	34 6

* … sen (1969)

† … of the *Subcommittee on Taxonomy of Staphylococci*

§ … ions in paranthesis

RESULTS

Table 1 lists the designation of patients and strains, the buoyant densities (BD) and mean denaturation temperatures (Tm) with standard deviation and per cent GC of each strain. Each result was calculated as the mean of at least two independent determinations.

All strains are found to have a guanine-cytosine content of their DNA within the interval recognized for typical staphylococci.

The per cent GC as calculated from the buoyant densities is for all strains found in the range of 33.4 to 34.8. This agrees with the results obtained by the Tm method (range 32.6 to 34.0 per cent GC), although this method appears to give slightly lower values. From Table 1 it is also seen that by both methods the per cent GC of the *Staphylococcus* strains is about 1 per cent GC lower than the per cent GC of the *Micrococcus* strains. This apparent difference was statistically tested and found to be significant ($p < 0.001$).

DISCUSSION

We have found that the strains identified as *Micrococcus* on the basis of the *Subcommittee's* test have per cent GC in the range typical of staphylococci (30–40). This confirms previous data by *Auletta & Kennedy* (1966), *Rosypal et al.* (1966) and *Mortensen & Kocur* (1967) who have also found strains identified as members of the genus *Micrococcus* to be in the low (30–40) per cent GC group.

The demonstration that such members of the genus *Micrococcus* associated with urinary tract infection are within the *Staphylococcus* per cent GC range is new. However, with the possible exception of *Micrococcus mucilaginosus* which has a GC content of 59 per cent (*Bergan et al.* 1970) pathogenicity has always been associated with a per cent GC in the [illegible]ge of 30–40, i.e. the *Staphylococcu*[illegible] a[illegible]ge.

The pathogen[illegible] a[illegible]d per cent GC of the strains of this study [illegible]upport the proposal by *Baird-Parker* (*Subcommittee* 1971) to place these organisms in a subgenus of the genus *Staphylococcus* and calls for a revision of the *Subcommittee's* decision (1971) to include organisms with widely differing GC contents in the genus *Micrococcus*. This is further supported by the observation that so-called *Micrococcus* strains capable of causing urinary tract infection show similarity to the staphylococci in cell wall composition (*Baird-Parker* 1970).

We realize that a suitable routine method for determining per cent GC or a test w[illegible] better correlation to per cent GC than [illegible] recommended by the *Subcommittee* is lac[illegible]ing. We prefer, however, in laboratory reports to use the name *Staphylococcus* for urinary isolates of the kind included in this st[illegible] [illegible] because that name will be in bet[illegible] [illegible]ance with established clinical use [illegible] *Staphylococcus* includes pathogenic [illegible] in contrast to the term *Micrococc*[illegible] [illegible] generally taken to indicate [illegible] organisms.

New is also our finding [illegible] correlation between per ce[illegible] GC [illegible] *Staphylococcus* range an[illegible] [illegible] ferences in glucose fer[illegible] sification based on co[illegible] per cent GC differen[illegible] teristics has been con[illegible] yellow pigmented stra[illegible] range of per cent GC [illegible] 1969). Definite taxo[illegible] clusions cannot be dr[illegible] tions until further [illegible] some indication of a [illegible] be found in the [illegible] (1966) which incl[illegible] GC content. On th[illegible] the data on [illegible] glucose fermen[illegible] published by [illegible] does not lend [illegible] cause of the d[illegible]

REFE[illegible]

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PURIFICATION OF VACCINIA HAEMAGGLUTININ

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Vaccinia haemagglutinin was isolated from HeLa cells and purified by a two step ultra centrifugation procedure In the first step the crude haemagglutinin was adjusted to 10 per cent sucrose and layered on top of 40 per cent sucrose Fractions containing haemagglutinin were adjusted to 50 per cent sucrose, and 40 per cent sucrose was layered on top of this The haemagglutinin which was recovered in the top fractions, contained no other proteins detectable by polyacrylamide electrophoresis and gave no precipitation line on double diffusion in agar against a vaccinia antiserum The density range in sucrose was 1 08 to 1 18 g/cm^3, indicating the lipoprotein nature of the material

Since *Burnet & Stone* (4) found that the vaccinia haemagglutinin (VHA) was distinct from the virion, several attempts have been made to purify this material *Youngner & Rubinstein* (12, 13) and *Anthony et al* (2) separated two haemagglutinins of different densities by centrifugating a homogenate of vaccinia-infected chorioallantoic membranes in a sodium chloride solution with a density of 1 063 g/cm^3 The lighter one was a non-specific tissue agglutinin, while the heavier one was virus specific, heat stable and resistant to trypsin *Neff et al* (9), examining a haemagglutinin from vaccinia infected HeLa cells, found a buoyant density in sucrose ranging from 1 11 to 1 21 g/cm^3 With other host cells different densities were found This made them suggest that the haemagglutinin is a hybrid consisting of a virus specific moiety (possibly a protein) and a host specific moiety (a lipid) *Marquardt* (7) examined a haemagglutinin preparation which he had extracted with organic solvents, and found that the agglutinating property was due to a lipid and the antigenicity being connected with a protein fraction

The aim of the present work is to isolate the VHA in a native state and to purify the material for further immunochemical studies

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MATERIALS AND METHODS

Virus and Tissue Culture

The vaccinia virus strain used was the Smallpox vaccine strain from Statens Seruminstitut, Copenhagen He La cells were obtained from Orion Oy, Helsinki, and were grown in Eagle s MEM supplemented with 10 per cent serum from newborn calves (Flow Laboratories Irvine Scotland) and antibiotics Rabbit lung cultures were obtained by trypsinization of lung tissue from young animals and the cells vere grown in the same medium as above

Virus Infectivity Titrations

The virus plaque technique was that of *Lindemann & Gifford* (5) as described earlier (1)

Immune Sera

For immunization of rabbits the virus was purified in the following way Partially purified vac

cinia was first propagated in rabbit lung tissue monolayer with 10 per cent rabbit serum (Flow Labs) added to Eagle s medium The harvested virus was thereafter grown in rabbit lung culture with 2 per cent serum obtained from the rabbit which was to be used for immunization A homo genate of infected cells (see preparation of crude VHA) was made and used for immunization after centrifugation to remove some cell debris Rabbits were immunized intravenously with one ml por tions of the centrifuged homogenate which con tained 2×10^5 plaque forming units (p f u)/ml Three injections were given at 3 week intervals

Haemagglutination (HA) Titration

Two-fold dilutions of haemagglutin were made in phosphate buffered saline (PBS 0 01 M phos phate, pH 7 2) in WHO perspex plates and mixed with equal volumes (0 25 ml) of 0 5 per cent vaccinia agglutinable chicken erythrocytes The titres were read after incubation at 37° C for one h

The haemagglutinin was considered virus specific when inhibited by the vaccinia antiserum but not by addition of normal calf serum

Haemagglutination Inhibition (HAI) Test

To two fold dilutions of immune serum were added 4 HA doses of haemagglutinin After in cubation for 20 min at room temperature chicken erythrocytes were added and the titre read after one h at 37° C Volumes of 0 25 ml were used of each reactant and all dilutions were made in PBS

Sonication

An MSE Mullard Ultrasonic disintegrator work ing at 15 KC was used and all treatments were made in an ice bath

Ultracentrifugation

The first centrifugation step was done with the SW 27 1 rotor (17 ml tubes) at 90 000 × *g* in a Beckman Spinco L265B Ultracentrifuge for 12 h and the second step with the SW 50 1 rotor (5 ml tubes) at 110 000 × *g* in a Beckman Spinco L50 Ultracentrifuge for 18 h Fractions were col lected by puncturing the bottom of the tubes or by pipetting off the top fractions after the flotation centrifugation The latter procedure was used for preparative purposes

Polyacrylamide Gel Electrophoresis

A Shandon apparatus was run according to the manufacturer s procedures The gels contained 7 5 per cent acrylamide polymerized by 0 003 M persulphate and 0 05 M N N N¹ N¹ tetramethyl ethylene diamine As reservoir buffer was used 0 05 M tris containing 0 26 M glycine and adjusted to pH 9 5 The electrophoresis was run with 3 mA on each tube The gels were stained with 1 per cent amidoblack dissolved in 7 per cent acetic acid and thereafter destained in the same solvent

Double Diffusion in Agar

The agar precipitation tests were done with 4 or 5 mm wells (10 mm and 5 mm distance respectively) with different agarose concentrations (0 4 0 7 and 1 0 per cent) in glycine buffer (0 1 M, pH 8 4)

Quantitation of Proteins

The protein content was determined by the Folin Ciocalteau method as described by *Lowry et al* (6) using bovine albumin as a standard

EXPERIMENTS AND RESULTS

Preparation of Crude VHA

Monolayers of HeLa cells were infected with vaccinia virus at a multiplicity of 10 p f u /cell The cells were harvested after 24 to 48 h by means of a rubber policeman By this time very little VHA was found in the medium, which was therefore discarded and the cells were suspended in an 0 1 M glycine buffer (pH 8 4)

It was early realized that isolated VHA was very unstable in solution and different buffers were tried as solvents The glycine buffer effectively stabilized the VHA The cell sus pension was frozen and thawed 3 times sonicated for 30 sec and then centrifuged for 10 min at 1,000×*g* to remove some cell debris The supernatant constituted the crude VHA

Preparation of Purified VHA

The crude VHA suspension was adjusted to 10 per cent sucrose, and 10 ml was layered on top of 7 ml 40 per cent sucrose in the glycine buffer Centrifugation was performed at 90,000×*g* for 12 h Fractions of 1 ml each were collected and examined for HA activity (Fig 1)

It is seen from Figure 1 that the bulk of the VHA was recovered in 2 fractions This step led to a concentration of the VHA and removal of most of the viruses Thus, the

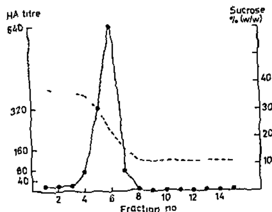


Fig 1 Centrifugation step I Crude VHA in 10 per cent sucrose was layered on 40 per cent sucrose and centrifuged for 12 hours at 90,000 × *g* The fractions are numbered from bottom to top ●——● HA titre – – – – sucrose

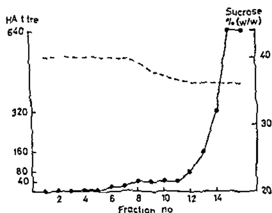


Fig 2 Centrifugation step II Partially purified VHA from centrifugation step I adjusted to 50 per cent sucrose was floated into a 40 per cent sucrose layer by centrifugation at 110,000 × *g* for 18 hours Symbols see legend to Fig 1

VHA fractions contained only 5×10^{0} p f u /ml as compared to 3×10^{9} p f u./ml in the crude preparation

The VHA material from the first centrifugation step was adjusted to 50 per cent sucrose and 40 per cent sucrose was placed on top of this After centrifugation for 18 h at $100{,}000\times g$, fractions of 0 3 ml were collected, and as seen from Figure 2 the VHA was recovered in the top fractions (Fig 2) The density range of these HA active fractions



Fig 3 Polyacrylamide electrophoresis of the VHA material obtained from the first (left) and second (right) centrifugation The protein bands were stained with amido black

was 1 08 to 1 18 g/cm³, and the combined fractions (no 14, 15, and 16), constituting the purified VHA, were practically free of viruses (2×10^{3} p f u /ml)

Some Properties of Purified VHA

VHA preparations from the 2 centrifugation steps, containing the same amount of VHA active material, were examined by electrophoresis in polyacrylamide gels (Fig 3) The VHA fraction from the first centrifugation showed several protein bands, while no visible band appeared after the second centrifugation step Some stained material was retained on top of the gel.

The protein content (Folin value) of purified VHA material with an HA titre of 640 was only 10 μg/ml The crude preparation and the VHA fraction from the first centrifugation contained 1 9 mg/ml and 3 1 mg/ml respectively

Purified VHA gave no precipitating line on double diffusion in agar against the anti-

vaccinia serum, nor was any precipitation visible in the ring test The VHA fractions with different densities were examined in the HAI test against the anti-vaccinia serum The same amount of serum was needed in order to completely inhibit 4 HA doses of each preparation

DISCUSSION

Little is found in the literature about purification of the poxvirus haemagglutinins Separation on DEAE cellulose (8) has been used as well as centrifugation procedures, but little is known about the properties of the isolated material Some investigations (7, 10) have been made with lipid extracts of vaccinia-infected cells

It is well known that vaccinia sensitive chicken blood cells are strongly agglutinated by lipids such as cardiolipin and lecithin We therefore omitted the use of organic solvent in order to reduce interference by non specific haemagglutinating lipids In this way we also aimed to isolate the VHA in the preformed state to avoid fragmentation by extraction procedures

In our hands the recovery of VHA after DEAE cellulose and Sephadex column chromatography was very low On this background we chose a 2-step centrifugation procedure The first step was performed to remove the bulk of the virus, thus giving some concentration of VHA and permitting lighter lipids to float over the VHA material The second step was performed to separate VHA from heavier lipoproteins and pure proteins

This procedure is in principle, similar to a membrane separation procedure (3, 11), and the density range of the product (1 08 to 1 18 g/cm³) is typical of lipoproteins

This defines our VHA preparation as a lipoprotein, possibly of membrane nature The purified preparation will be subjected to further immunochemical studies

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A COMPARISON OF ANTIGENIC STRUCTURE AND PHAGE PATTERN WITH BIOCHEMICAL PROPERTIES OF *STAPHYLOCOCCUS AUREUS* STRAINS ISOLATED FROM HARES AND MINK

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Strains of *Staphylococcus aureus* isolated from the nares of hares and mink were tested with the basic sets of human and bovine phages and with factor sera prepared against human staphylococci Of 21 hare strains the 17 which belonged biochemically to the D biotype agglutinated strongly in h_2 factor serum and were lysed by group II phages Four hare strains and all 108 mink strains which belonged biochemically to the E (canine) biotype, were not lysed by the phages even at RTD × 1,000 Serologically the four hare strains (probably of canine origin) contained the a_5 antigen whereas only 17 per cent of the mink strains were typable exhibiting weak reactions and no definite pattern

The epizootiology of staphylococcal infections and the biological properties of the isolated strains have been studied far less frequently in small, especially wild, animals than in cattle, sheep, swine and poultry In some species of the small mammalia the carriership of staphylococci is rather high and staphylococcal infections are not uncommon The reported epizootics of staphylococcal suppurative dermatitis in mink (2, 3) and the fatal septicaemia following urolithiasis (11 25) are remarkable Disseminated staphylococcal infections are also well known in hares and wild rabbits (1, 5, 19) A few reports have been given of the occurrence of pathogenic staphylococci in healthy mink and hares and of the physiological properties of the strains (4, 8, 10, 22, 23)

In the present paper the sensitivity to phages and the serological typability of staphylococci isolated from the nares of mink and hares have been determined and compared with the biochemical properties reported earlier (8, 10)

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MATERIAL AND METHODS

From the nares of 314 healthy mink (*Lutreola lutreola*) bred in two farms in Northern Moravia 107 strains of *Staphylococcus aureus* were isolated and from the nares of 462 hares (*Lepus europaeus*) killed in the Olomouc region 21 strains In addition one strain isolated from the viscera of a

TABLE 1 *Serological and Phage Typing of 21 S aureus Strains Isolated from Hares*

Biotype	No of strains	Phage pattern	Phage group	Serological pattern	Total
D	17	3A/3C/55/71/116	II	h_2	17
E	4	NT		a_5	2
				$a_5/e/h_1/h_2/k_1k_2$	1
				$a_5/e/h_1/k_1k_2/n/263$ 1	1

mink suffering from urolithiasis and staphylococcal septicaemia was examined

Phage typing was carried out with the phages of the basic set for typing human staphylococci (26) and with the basic set for typing bovine staphylococci (27) Strains not typable with the routine test dilution (RTD) were retested with RTD × 1,000

Slide agglutination was performed according to the method of *Oeding* (15) For technical details see also (13) All antisera were prepared by immunization of rabbits with human *S aureus* strains The hare strains were typed with factor sera a_4, a_5 b_1, c_1, e, h_1 h_2, i_1i_2, k_1 k_1k_2 m n *263–1, 263–2* Of the 108 mink strains, 26 selected strains were typed with the complete set of factor sera whereas the remaining strains were tested with factor sera c_1 h_2 k_1k_2 and m only The presence of the precipitinogens polysaccharide Aβ and protein A was tested by double diffusion in agar gel according to (12, 6)

The biochemical tests used to characterize the strains were reported in (8, 10)

RESULTS

Of the 21 hare strains 17 were found to belong to the D biotype and 4 to the E biotype of *Hajek & Maršalek* (8, 9) The biotype D strains coagulated human but not bovine plasma, they were of crystal violet type C (violet) and usually non pigmented produced β haemolysin but neither α haemolysin nor fibrinolysin The characteristics of the biotype E strains are given below

All biotype D strains agglutinated strongly in the h factor serum and had the phage pattern 3A/3C/55/71/116 (Table 1) The 4 biotype E strains all contained the type agglutinogen a_5, 2 of them other agglutinogens in addition None of the 4 strains were typable by phage, even at RTD × 1 000

All the 108 mink strains coagulated bovine but not human plasma, they were of crystal violet type E (white), produced β haemolysin but not α haemolysin, fibrinolysin or pigment They were classified as biotype E (9, 10)

Of the 26 mink strains which were tested serologically with the complete set of factor sera, 10 strains were typable Of the remaining 82 strains, which were tested only in 4 factor sera, 8 strains were typable The prevailing agglutinogens were c_1 m and h_2 (Table 2) Thus only 17 per cent of the

TABLE 2 *Serological and Phage Typing of 108 S aureus Strains Isolated from Mink*

Biotype	No of strains	Phage typing	Serological pattern*	Total
E	108	NT	a_4/m	1
			a_5/h_2	1
			c_1	3
			c_1/m	1
			h_2	7
			h_2/m	1
			i_1i_2	1
			m	3
			NT	90

* 26 strains tested in complete set of factor sera 82 strains in factor sera c_1 h_2 k_1k_2 and m only

mink strains could be typed serologically The strain isolated from the case of septicaemia gave a strong c_1 agglutination whereas all the other agglutinations were weak No mink strain was typable by phage, even at RTD × 1,000

Polysaccharide Aβ was demonstrated in all except one of the hare biotype D strains but in only one of the four hare biotype E strains All the mink strains produced a precipitation line with serum Wood 46 which reacted with the polysaccharide A line No strain had protein A

DISCUSSION

The majority of the *S aureus* strains isolated from hares were very homogeneous with regard to biochemical properties and phage and serological typing They were placed in the D biotype (8, 9), were lysed by phages of group II and contained the h_2 agglutinogen only The demonstration of only one type agglutinogen in the hare biotype D strains is in accordance with the finding that animal staphylococci contain few of the agglutinogens found in human strains (14, 16, 17, 18) The h_2 agglutination was, however, strong *Pulverer* (22) and *Pulverer* & *Entel* (23) have reported staphylococci isolated from the upper respiratory passages of hares with corresponding properties and sensitivity to phages of group II

Four hare strains and all the mink strains had the same biochemical characteristics and were placed in the E biotype (8, 9, 10) Strains with corresponding biochemical properties have been found to be the most usual type in dogs (7) and have also been isolated from the nares and infected lesions of wild mink (3 4) All the present strains belonging to the E biotype were resistant to the standard human and bovine phages This is in accordance with the findings of other authors (2 3 4) Serological typing clearly distinguished the hare biotype E strains from the mink strains The 4 hare strains were easily typable all containing the agglutinogen a_1 In contrast to this only 17 per cent of the mink strains were typable, producing weak agglutination reactions without a characteristic pattern The only mink strain giving a strong agglutination was isolated from an infection

The properties of the 4 hare biotype E strains corresponded very well with those of a collection of canine strains earlier reported (7, 16) They had the same biochemical properties, were non typable by phage and contained a_1 as the major agglutinogen These data indicated that pointers were the source of the 4 biotype E strains isolated from hares

In order to achieve a better typability of animal strains of *S aureus* it will be necessary to search for phages and antigens characteristic of the biotypes Absorbed antiserum prepared against a canine strain has with some success been used for the typing of strains isolated from dogs (20, 24, 16), and absorbed antiserum prepared against a mink strain for the typing of strains isolated from mink (21) The absorbed sera agglutinated the majority of canine strains, respectively mink strains, but not strains isolated from other animal hosts

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PURIFICATION AND CHARACTERIZATION OF SEROLOGICALLY ACTIVE CELL WALL SUBSTANCES FROM *PLANOCOCCUS* STRAINS

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From the cell walls of four *Planococcus* strains polysaccharide and murein were separated and purified The walls contained small amounts of polysaccharides, murein being the major constituent Teichoic acids were not present Both chemically and serologically the polysaccharides of two strains seemed to be identical whereas this polysaccharide and those from the two other strains all were clearly different Each of the three types of polysaccharides produced a specific precipitation line in agar gel diffusion Quantitative agglutination with anti-murein antisera revealed that *Planococcus* mureins share antigenic determinants with *Staphylococcus* mureins but also have determinants of their own, which seem to be shared by *Planococcus* strains

Recently, *Kocur et al* (2, 3, 4, 12) demonstrated that the per cent GC content of flagellated Gram-positive cocci of marine origin was different from that of micrococci They suggested that flagellated marine cocci should be transferred from the genus *Micrococcus* to the genus *Planococcus Schleifer* & *Kandler* (18) found that the murein of these cocci was clearly different from the mureins of micrococci and staphylococci Serological examination of whole cells (*Oeding*, 14) indicated that the strains of marine cocci examined were heterologous with regard to their antigens

The purpose of the present investigation was to isolate and characterize serologically active cell wall substances from strains of flagellated marine cocci

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MATERIALS AND METHODS

Strains The 12 strains of flagellated, marine cocci were kindly furnished by *M Kocur*, Czechoslovak Collection of Microorganisms, Brno

Preparation of cell walls Cell walls were prepared from the four strains CCM 1849, 2069, 2104 and 2389 Appr 10 g of wet bacteria were disrupted in the X press, × 5 passages −25° C The walls were purified as described in (21)

Isolation and purification of polysaccharide Cell walls were extracted with 10 per cent trichloroacetic acid (TCA) at 4° C for 24 h (1) The extracts were dialyzed against 0 02 M phosphate buffer, pH 7 2 and applied to DEAE cellulose (Eastman) columns (2 4 × 40 cm), equilibrated with the same buffer Elution was performed with 0 02 M phosphate buffer, pH 7 2 using a gradient system from 0 to 1 M KCl The flow rate was appr 40 ml/h Fractions were collected and the optical density read at 280 nm. The fractions found to be positive with homologous immune serum in the ring test were pooled, dialyzed and freeze dried Further purification was performed by gel filtration on Sephadex G-75 columns (2 6 × 95 cm) using a 0 1 M Tris-HCl buffer, pH 8 0, with 0 5 M KCl

The purified polysaccharides were examined for protein contamination by polyacrylamide gel electrophoresis with sodium dodecyl sulphate (SDS) (20)

Isolation of murein The murein was obtained from the TCA insoluble portion of the four cell wall preparations, using the method described in (15 8)

Analytical methods Circular paper chromatography was performed as described earlier (5) Four solvent systems were used

1 Butanol Pyridine H_2O – (6/4/3 v/v)
2 Ethylacetate Pyridine H_2O – (40/11/6 v/v)
3 Butanol Ethylalcohol H_2O NH_3 d 0 88 – (40/11/49/1 v/v)
4 Propanol NH_3 d 0 88 – (6/4 4 v/v)

Reducing sugars amino sugars and sugar alcohols were localized with alkaline silver nitrate reagent (19) Reducing sugars were also detected with aniline – phthalate spray (16)

Quantitative determination of carbohydrates as trifluoroacetylated derivatives of the corresponding alditols was carried out by GLC as described in (10) and by *Endresen* (in press) Hexosamines were determined by a modified Morgan Elson method (11)

Quantitative analysis of amino acids was performed by GLC using the technique described in (17)

Periodate oxidation One mg of polysaccharide material in 1 ml of 0 1 per cent sodium periodate was incubated at 37° C for 2 h and then dialyzed for 24 h against phosphate buffered saline (PBS)

Digestion with trypsin The enzyme reaction was carried out in 0 2 M phosphate buffer pH 7 4, at 37° C The enzyme/substrate ratio was 1/100 by wt

Serological methods Immune sera were produced against formalin killed bacteria by intravenous injections (13) into New Zealand white rabbits of the Institute s breed Immune sera against isolated murein were produced by one intramuscular injection of 6 mg of murein in saline emulsified in an equal volume of Freund s incomplete adjuvant followed by one intravenous injection of 2 mg of murein after 8 weeks

Double diffusion in agar slide agglutination of live bacteria and tube agglutination of murein were performed as described earlier (7 13 6) Quantitative agglutination was performed as described in (9) Immunoelectrophoretic analyses were performed using an LKB apparatus as described by the manufacturer (LKB Produkter)

RESULTS

Polysaccharide

When whole bacteria of the strains 1849, 2069, 2104 and 2389 were tested in agar gel against the homologous immune sera one strong and usually also one faint precipitation line appeared The faint line was not produced even in high concentrations (5 mg/ml) of purified cell walls The antigen corresponding to the strong line was extracted almost completely by 10 per cent TCA in the cold The yield of the crude precipitinogen was appr 11-14 per cent of the dry weight of the cell walls for the strains 1849, 2069 and 2389, and 5 per cent for the strain 2104

Further purification was performed on DEAE cellulose and Sephadex G 75 columns Serologically active material from the strains 1849 and 2069 was eluted from the Sephadex columns close to the void volume (K_{av} = 0 15 0 20) The polysaccharides from the strains 2104 and 2389 seemed to be of smaller size (K_{av} = 0 45 0 50)

On double diffusion in agar gel the polysaccharide 1849 and 2069 lines fused completely whereas the polysaccharides from the strains 2104 and 2389 reacted in the homologous sera only On immunoelectrophoresis in agar the 1849 and the 2069 polysaccharide moved identically towards the cathode whereas the 2389 and the 2104 polysaccharides migrated towards the anode

Trypsin digestion of the polysaccharides did not affect the serological activity Oxidation with periodate had no influence on the serological activity of the 1849, 2069 and 2104 polysaccharides, whereas in the 2389 polysaccharide two precipitation lines appeared in agar gel diffusion The line nearest to the serum well fused with the precipitation line of the untreated antigen Further oxidation did not change the result

The polysaccharide preparations were tested for impurities by SDS polyacrylamide gel electrophoresis using appr 50 µg of substance per tube Two or three faint bands were observed indicating a low contamination with protein like materials

The results of the chemical analyses of the purified polysaccharides are shown in Table 1 The data for the 1849 and 2069 polysaccharides were almost identical and differed

TABLE 1 *Chemical Composition of the Purified Polysaccharides Isolated from Four Planococcus Strains*

Polysaccharide	1849	2069	2104	2389
		µmol/mg*		
Mannose	0 16	0 14	trace	0 21
Glucose	0 92	0 88	trace	0 28
Galactose	0 30	0 34	trace	0 55
Hexosamines	0 54	0 58	1 70	0 84
Glucosamine	+	+	+	+
Galactosamine	+	+	–	–
Phosphorus	0 16	0 16	0 33	0 50
Alanine	0 27	0 31	0 46	0 55
Glutamic acid	0 19	0 18	0 33	0 21
Lysine	0 14	0 11	0 16	0 14
Aspartic acid	0 33	0 35	0 09	0 02
Threonine	0 11	0 09	0 06	<0 01
Serine	0 15	0 16	0 05	<0 01
Valine	0 09	0 11	<0 01	<0 01
Ileucine	0 07	0 05	0 14	<0 01
Leucine	0 16	0 12	0 12	<0 01
Glycine	0 12	0 08	0 11	<0 01
Muramic acid	+	+	+	+

* Mean of three analyses

significantly from those for the 2104 and 2389 polysaccharides, which again differed from each other The 2389 polysaccharide had only traces of amino acids in addition to those known to be present in the murein, *i e* alanine, glutamic acid and lysine

All the preparations contained glucosamine and the 1849 and 2069 polysaccharides apparently also contained galactosamine Muramic acid was detected in all the hydrolysates by paper chromatography Hexoses were present in variable amounts, but ribitol or glycerol could not be detected by paper or GLC The phosphorus content was rather low from 0 5 to 1 5 per cent

Murein

The TCA insoluble materials from the cell walls were digested with trypsin dialyzed and freeze dried Quantitative amino acid analyses by GLC gave results corresponding to those found by *Schleifer & Kandler* (18)

Murein constituted 65 80 per cent of the dry weight of the cell walls

Murein from the four *Planococcus* strains and from strains of staphylococci were tested for agglutination in immune sera prepared against the *Planococcus* 1849 and 2389 mureins The two sera gave almost identical results The agglutinin titres for the *Planococcus* and *Staphylococcus* mureins were 1 128 and 1 32 respectively

The results of quantitative agglutinin determinations between the 2389 anti murein antiserum and suspended mureins isolated from the *Planococcus* strains and from *S aureus* Copenhagen are shown in Fig 1 The data indicate that in addition to antigenic determinants shared with the *Staphylococcus* murein the *Planococcus* mureins have other determinants apparently identical in these *Planococcus* strains

Serological Testing of 12 Planococcus Strains

When live bacteria of the 8 remaining *Planococcus* strains were tested against the four antibacterial immune sera, none of these strains produced any of the three specific precipitation lines described above Agglutination revealed certain cross reactions but did not permit reliable conclusions

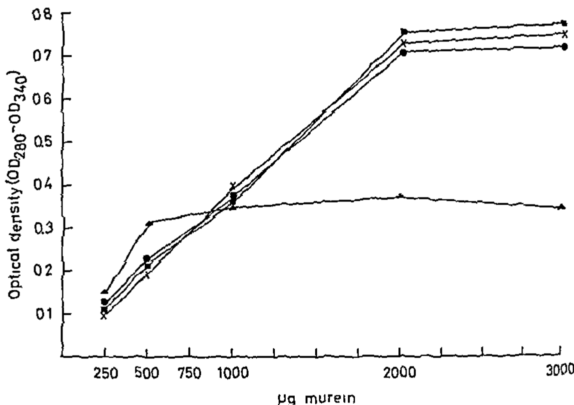


Fig 1 Quantitative agglutinin reactions between sonically disrupted mureins of *Planococcus* 1849 (×——×), *Planococcus* 2104 (●——●), *Planococcus* 2389 (■——■), *S aureus* Copenhagen (▲——▲), and *Planococcus* 2389 anti murein antiserum

DISCUSSION

Serologically active polysaccharide was extracted apparently quantitatively by TCA from the walls of four *Planococcus* strains However, the amounts extracted were small showing that the walls of these strains have only minor quantities of polysaccharide material Analyses of the purified polysaccharides revealed that teichoic acids were not present Both chemically and serologically the polysaccharides isolated from the 1849 and 2069 walls appeared to be identical These polysaccharides were definitely different from the wall polysaccharide of strain 2104 and from that of strain 2389 In agar gel the three types of polysaccharide produced precipitation lines which did not cross react Also in their elution from Sephadex columns and in their mobility in electrophoresis the 1849 and 2069 polysaccharides differed from the other two polysaccharides

These results and the examination of the 8 remaining strains by agglutination and precipitation show that *Planococcus* strains are heterogeneous with regard to the composition of their wall polysaccharides and agglutinogens, thus confirming the earlier report of *Oeding* (14) According to the per cent GC content and some other criteria *Kocur* (personal communication) has separated 12 *Planococcus* strains into four groups Of the four wall polysaccharides here examined, 1849 and 2069 are the only ones belonging to *Kocur's* group 1, strain 2104 being placed in group 2 and strain 2389 in group 3 So far this differentiation agrees with the present chemical and serological examinations of the polysaccharides, but the serological examina-

tions of the additional strains do not indicate the presence of a shared wall polysaccharide within each group

Murein was found to constitute the bulk of the cell wall material, i e 65-80 per cent Quantitative amino acid analyses of the isolated mureins revealed that alanine, glutamic acid and lysine were present in quantities corresponding to those reported by *Schleifer* & *Kandler* (18) Agglutination with murein immune sera showed that *Planococcus* mureins share antigenic determinants with *Staphylococcus* murein Cross reactions between *Staphylococcus* and *Micrococcus* murein have earlier been demonstrated by *Helgeland* & *Grov* (8) The sugar chains and similarity in the tetrapeptide may be the explanation of the cross-reactions Specific antigenic determinants apparently shared by the *Planococcus* mureins investigated were also demonstrated

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THE INFLUENCE OF GROWTH TEMPERATURE ON THE THERMAL DENATURATION OF RIBOSOMES ISOLATED FROM *ESCHERICHIA COLI*

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The thermal stability of ribosomes isolated from *Escherichia coli* grown in minimal medium at different temperatures has been determined by measuring the hyperchromicity at 260 nm while gradually increasing the temperature of the ribosomes which were suspended in standard buffer containing 0 01 M Mg^{++} ions The melting profiles are dependent upon the sample position in the spectrophotometer since the ribosome suspensions become turbid as the temperature is increased The measurements did not show any difference between the T_m values obtained for ribosomes isolated from cells grown at 15° C and 37° C respectively but at temperatures above 71° C the denaturation profiles of 70 S ribosomes from *E coli* grown at 15° C and 37° C are different

The thermal stability of ribosomes isolated from a variety of psychrophilic, mesophilic and thermophilic organisms has been examined and found to correlate with maximum growth temperature of the organisms when they are grown at their respective optimal temperatures (*Pace & Campbell* 1967) In addition cells from the one organism grown in the same medium, but at different temperatures have been shown to have constant proportions of RNA, DNA and protein (*Hansen* 1971 *Schaechter et al* 1958) If cell division takes place at low incubation temperatures free 70S ribosomes will accumulate at the expense of polysomes while the ribosomal subunits remain almost unchanged (*Uchida et al* 1970)

It was therefore of interest to examine the heat stability of ribosomes from an organism grown at different temperatures The ribosomes have been characterized by their thermal denaturation profiles Thus, this paper describes the influence of growth temperature on the heat stability of ribosomes isolated from *E coli* in balanced growth at 37° C and 15° C, respectively

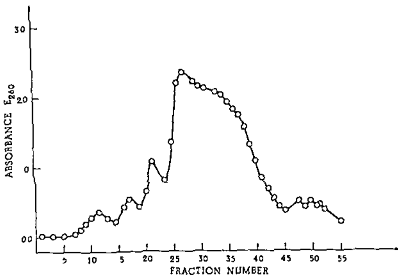
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MATERIALS AND METHODS

Organism and Growth Conditions

E coli K 12 Su 65/42 was grown in minimal medium (*Hansen et al* 1971) with 0 2 per cent glucose as carbon source The cultures were grown in a Biotec fermentor Type Fl 110 (LKB House 232 Addington Road South Croydon CR 2 8 YD) as described by *Bitsch et al* (1972) and under the same conditions as described by *Hansen* (1971)

Cells were harvested during balanced growth from 30 l of medium with 100 μg dry weight per ml They were washed three times with 20 ml cold TM (10 ²M tris HCl pH 7 3 with 10 M $MgCl_2$)



F g 1 Sucrose dens ty grad ent cen r fugat on prof le of r bosomal part cles obta ned from *Escherichia coli*

lyoph l zed and stored n sealed ampoules at —20 C As the resul s can be repeated with ribosomes from cells wh ch have not been lyophi lized this method of preservation is appl cable to the present stud es

Af er having collected cells grown at 37° C tl e fe mentor was nearly emp ed tl e temperature was mmed ately changed to 15 C and the re ma n ng culture was d luted with f esh med um until a bacter al dens ty of 20 μg dry we ght per ml was obta ned Cells grown at 15 C were also harves ed from 30 l of med um w th 100 μg dry we ght per ml and trea ed exactly as the cells grown a 37 C

The tempera ure was again changed to 37 C and mo e cells were collec ed at that tempera ure Subsequently a further batch at 15° C was grown and the cycle vas repeated unt l three batches of cul ure grown at each ncubat on temperature had been collected

No lag phase appeared dur ng the exper ment nd cat ng that no spontaneous temperature-depen dent mutant had developed

P epa at on of tl e C ude R bo omal Fract on

It appl es to all the isolat on procedures that the temperature was ma nta ned at 4 C Approxima e ly 0 5 g of lyoph l zed cells were suspended n 20 ml of TM Cells were broken by two maximum power 30 sec pulses of son c osc l at on n a MSE 100 watt ultrason c d s ntegrator (Measuring & Sc en f c Equ pment LTD 25 28 Buck ngham Gate London SW 1 England) Cell debris was removed by 15 min centrifugat on in a MSE 18 H gh Speed centrifuge operat ng at 25 000 g Ribosomes were then pelleted by 2 h centrifugat on at 105 000 g n a MSE Superspeed 65 centr fuge

The pellet was suspended n 10 ml of TM placed n V sk ng 8/32 tubes and d alysed o er night aga nst 1000 ml of TM Accumulated debris was removed by low speed centrifugat on upon wl ch the supernatant was subjected to zonal cen trifugation

Zonal Centr fugat on

All runs were carr ed out at 4° C A 440 g grad ent of sucrose (n TM) rang ng from 10 to 20 per cent (w/w) was l nearly ntroduced into a MSE B XIV ro or wh ch was runn ng at 2 500 rev/min n a MSE Superspeed 65 centrifuge The pump ng rate was 10 ml/m n (B otec LP 600) The grad ent was followed by an underlay of 50 per cent (w/w) sucrose n TM wh ch was added unt l the lead ng edge of the grad ent appeared in the core tube A 10 ml sample was introduced through the core l ne and as followed by an overlay of 100 ml TM The pump ng rate was 35 ml/m n (Hughes H loflow F A Hughes & Co L m ted Blenheim Road Longmead Epsom Surrey) The rotor speed was then ra sed to 30 000 rev/m n and held for 4 h af er wh ch t was reduced to 2 500 rev/m n when the preparat on n the rotor was d splaced (at 2 500 rev/m n) by pump ng n more underlay (10 ml/m n) The effluent passed through a flow cell of 1 cm path length mounted n a Beckmann DB G spectrophotometer (Beckmann Instruments GMBM Munchen 45 Frankfurter R ng 115) and ts ext nct on at 260 nm was

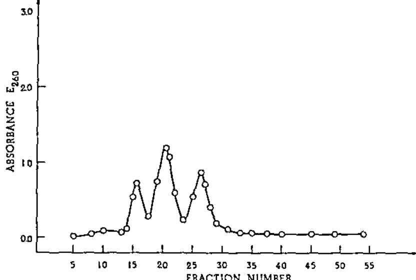


Fig 2 Sucrose density gradient centrifugation profile of 70S peak after dialysation against distilled water

recorded on a 10" Beckmann chart recorder It was then collected in 10 ml fractions in a fraction collector (LKB UltroRac Type 7000, LKB Stockholm, Bromma 1, Sweden) modified to change at one minute intervals

Isolation of 70S, 50S and 30S Particles

The bi phasic peak reaching from fraction 27 to 37 (Fig 1) consists of 70S and 100S The 100S is a dimer of 70S, and there exists an equilibrium between two 70S particles and one 100S particle (*Britten & McCarthy* 1959) This equilibrium is totally displaced in favour of 70S on lowering the Mg^{+} concentration and ribosome concentration As is seen, the 100S and 70S fractions dissociate into 70S 50S and 30S after dialysation against water (Fig 2) The peaks around fraction 11 in Fig 1 are probable degredation products whereas the peaks around fraction 50 can be polysomes or debris which have not been removed by low speed centrifugation

The fractions numbers 27–37 (Fig 1) were collected diluted 1 1 (v/v) with TM and spun in a MSE Superspeed 65 centrifuge at 150,000 g to pellet ribosomes One fraction of the pellet was suspended in 2 ml of TM and dialysed overnight as described above Debris was removed by low speed centrifugation and the 70S suspension left was used for the thermal denaturation determinations

30S and 50S subunits we e obtained from the remaining pellet which was suspended in 8 ml of distilled water, dialysed overnight against 1000 ml of distilled water and then subjected to zonal centrifugation conducted as previously described except that distilled water was used instead of TM for the gradient preparation and as overlay The 30S (fraction numbers 15 and 16, Fig 2) and 50S (fraction numbers 18–22, Fig 2) preparations were collected and handled as described for 70S

Thermal Denaturation Profiles

The ribosome suspensions were diluted with TM until their absorbance at 260 nm was 0 45–0 55 and then evacuated to prevent the formation of air bubbles during heating

As the melting profiles described by plotting temperature against the relative absorbance are independent of the concentration of ribosomal particles, the only reason for choosing this concentration is that it causes an increase in absolute absorbance of a size suitable to establish the melting profiles with acceptable accuracy Absorbance was measured with a Pye Unicam SP 1800 (Pye Unicam Ltd , York Street, Cambridge CB 1 2P X, England) spectrophotometer (using a 1 cm cell) equipped with two temperature controlled cell holders (SP 870 and SP 871) each of which were placed in the normal position (n s p), i e the cuvette being far from the photo electric cell, and in the second sample position (2nd s p), i e the cuvette being close to the photo-electric cell The cell holders were connected to a circulating bath which could produce programmed temperature sequences (Heto Ol PG 623, Birkerod Denmark) The spectrophotometer output was recorded on a Servogor RE 511 chart recorder (Goerz Electro Ges m b H A-1101 Wien)

The temperature was measured directly in the cuvette with a resistance thermometer (*Mettler*

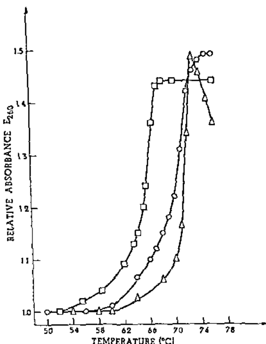


Fig 3 Thermal denaturation profiles obtained in n s p ○ 70S △ 50S, □ 30S specimens isolated from *Escherichia coli* grown at 37° C

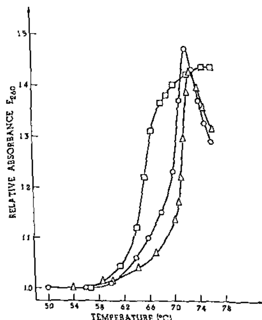


Fig 4 Thermal denaturation profiles obtained in n s p ○ 70S △ 50S □ 30S specimens isolated from *Escherichia coli* grown at 15° C

TM 101, CH 8606, Greifensee, Zurich) and was monitored continuously on a recorder (*Servogor* RE 511)

Each sample was heated from 35°–75° C at a rate of ½° C/min In this range the temperature gradient measured directly in the cuvette was constant to within 0 5 ± 0 01° C/min

The necessity of keeping the temperature gradient constant and reproducible has been demonstrated by some experiments which showed that the T_m values become higher accordingly as the heating rate is increased which probably indicates that the denaturation of the ribosomal particles is a reaction dependent on time as well as on temperature The T_m values (the temperature at the midpoint of the maximum hyperchromicity) were constant to within ± 0 5° C

RESULTS

Figures 3 and 4 present the data obtained when the samples were heated in the normal sample position in the spectrophotometer, Figures 5 and 6 presenting the analogous data for samples heated in the second sample position The results have been reproduced in three independent experiments

The differences between the absorbances measured in the two positions is an expression of the turbidity of the sample, since the measured absorbance in n s p is due partly to light scattering which in 2nd s p only contributes little because of the shorter distance from the cuvette to the photo electric cell

There is also a tendency for the T_m *values* from n s p measurements to be larger than those obtained at the 2nd s p This is most pronounced in the case of 70S and 30S samples (Table 1)

The profiles show that there is a fall in E_{60} at the 2nd s p (Fig 5 and 6) after maximum hyperchromicity has been obtained This *is also observed at the n s p* of 50S samples from both culture types and of 70S samples from 15° C cultures (Fig 3 and 4) 50S profiles measured in the 2nd s p are distinctly different, almost reaching the baseline at the last three degrees

Thus it is evident that shifts, T_m values and profiles are dependent upon the position of the samples in the spectrophotometer

TABLE 1 T_m values and Shifts for Ribosomes and their 50S and 30S Subunits Isolated from Escherichia coli Grown at 37° C and 15° C, Respectively

Heated at	Ribosomal particle	Growth temperature 37° C		15° C	
		T_m in ° C	Shift*	T_m in ° C	Shift*
n s p	30S	65 4	44	65 5	44
	50S	71 4	48	71 1	42
	70S	70 4	51	70 2	47
2nd s p	30S	65 0	27	64 5	29
	50S	71 3	33	70 1	24
	70S	68 2	32	67 8	29

* The maximal hyperchromic effect in per cent

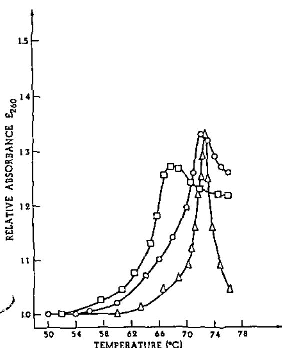


Fig 5 Thermal denaturation profiles obtained in 2nd s p ○ 70S △ 50S □ 30S specimens isolated from *Escherichia coli* grown at 37° C

50S particles melt out very little at low temperature and exhibit large hyperchromicity in a narrow temperatur range, whereas 30S particles melt out at lower temperatures and over a broader range

When the samples are in the second position, no differences have been observed between the melting profiles of analogous particles prepared from 37° C and 15° C cultures respectively (Fig 5 and 6) But the two 70S curves obtained in normal sample position differ significantly if the heating is continued after the shift is obtained Also E_{260} remains constant upon further heating of 70S

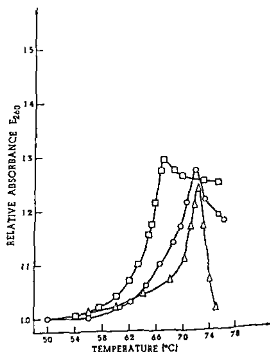


Fig 6 Thermal denaturation profiles obtained in 2nd s p ○ 70S △ 50S □ 30S specimens isolated from *Escherichia coli* grown at 15° C

samples from 37° C cultures while it falls for 70S specimens from 15° C cultures

DISCUSSION

The predominant factor influencing the thermal stability of ribosomes is thought to be the packing arrangement and interaction between rRNA and ribosomal proteins (*Mangiantini et al* 1962, *Stenesh & Holazo* 1967) This may be due to the RNA and protein compositions and structures (*Saunders & Campbell* 1966) and to the existence of different stabilizing factors (*Friedman et al* 1967)

The larger hyperchromic effect (approx 50 per cent) for ribosomes relative to rRNA (approx 25 per cent) has been found in earlier investigations (*Elson* 1959, *Mangiantini et al* 1965) to be higher that that induced by simple loss of helical structure and it has been proposed that it may be due to depolimerization of rRNA by a latent ribonuclease (attached to the ribosomes) This should be activated by disruption of the RNA protein complex during heating However, the investigations described here indicate that the large hyperchromic effect obtained in the case of 70S ribosomes is due to the higher degree of light scattering for denatured ribosomes

Comparison of the melting profiles obtained in the normal and second sample positions show that the shifts (Table 1) are larger for n s p -measurements because the samples become turbid during denaturation The samples have the same absorbance in each position before heating, so that the profiles obtained in the 2nd s p reflect to a greater extent the hyperchromicity resulting from RNA and protein disruption than those obtained in the n s p which are most effected by the turbidity

This conclusion is supported by the melting profiles recorded from samples containing 0 1 per cent diethylpyrocarbonate, $(C_2H_5O)_2CO$, (an effective RNase inhibitor) (*Wolf et al* 1970) which showed even higher shifts than those for 70S ribosomes measured in the n s p

The differences in melting profiles of 70S, 50S and 30S particles may be due, among other things, to differences in base composition from 16S and 23S The results presented here correspond with those obtained in *in vivo* experiments by *Sogin & Ordal* (1967) who showed that in the case of *Staph aureus,* the 16S RNA from 30S subunits was more heat labile than the 23S from 50S subunits

These investigations show that there is no difference between the T_m values obtained for the 30S, 50S and 70S particles prepared from cells grown at 15° C and 37° C, respectively, but a difference between the profiles obtained in normal position for 70S specimens prepared from 15° C cultures and 37° C was observed

This difference consists in a drop in E_{260} by heating the 70S ribosomes from 15° C cultures to temperatures higher than 71° C, while E_{260} for 70S ribosomes from 37° C remains constant during this heating A drop in E_{260} has also been observed by *Pace & Campbell* (1967)

The drop in E_{260} at high temperatures is probably due to the formation of particles of denatured ribosomes *Duysens* (1956) has shown that a given amount of material has a lower absorption when it is suspended than when it is dissolved The drop in absorption is counteracted by the light scattering of the formed particles, which explains the more shallow curves obtained in n s p compared to 2nd s p An interpretation of the difference seen in n s p of the 70S ribosomes from cells grown at 15° C and 37° C requires further experiments

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IDENTIFICATION OF SMALL AMOUNTS OF ANTIBIOTICS BY ELECTROPHORESIS AND BIO-AUTOGRAPHY

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The use of agar gel electrophoresis and bio autography for the identification of small amounts of 12 different antibiotics, when present in a mixture, has been studied The procedure for the electrophoretic separation of the components is given The antibiotics are detected by means of various microorganisms of high sensitivity to each of the components The limits of detection obtained for the various antibiotics were in the range from 0 25 to 50 μg/ml

The separation of antibiotics by thin-layer chromatography or gel electrophoresis followed by bio-autographic visualization of the separated components has been reported by several authors (1, 4, 7, 8) Most of the methods involve the application of antibiotic solutions of high potencies In order to obtain a method of higher sensitivity, the procedures described by *Lightbown & de Rossi* (5) and *Marten* (6) were tried out After the electrophoresis in agar gel, this was covered with a second layer of agar seeded with a test organism However it seemed difficult to obtain thin layers of uniform thickness which is essential to a reproducible and highly sensitive method Due to the long duration of the electrophoresis (1 to 3 hours), the growth of unwanted microorganisms often occurred Further, after each electrophoretical run, only one test organism could be used and none of the suggested organisms were sufficiently sensitive to all the antibiotics under examination The method described in this work includes 30 minutes of electrophoresis and it permits the identification of several antibiotics simultaneously normally giving a better sensitivity than described in the literature

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MATERIAL AND METHODS

Antibiotics Examined

The origins and the potencies of the antibiotic standard preparations used are shown in Table 1 which also describes the preparation of stock solutions These were stored in a refrigerator and used within 7 days of preparation (Any possible loss of antibiotic activity by this storage was too small to be registrated by the present method)

Reagents

Phosphate buffer solution, pH 6 5 22 g of potassium monohydrogen orthophosphate and 28 g of potassium dihydrogen orthophosphate were dissolved in 1 litre of distilled water

Phosphate buffer solution, pH 8 0 48 g of sodium monohydrogen orthophosphate dihydrate and 2 g of potassium dihydrogen orthophosphate were dissolved in 1 litre of distilled water

Electrolyte pH 6 5 25 ml of the phosphate buffer solution pH 6 5, were diluted to 1 litre with distilled water

TABLE 1 *Antibiotic Standard Preparations and Stock Solutions*

Antibiotic	Source	Potency per mg	Potency of stock solution per ml of distilled water
Bacitracin	2nd International Standard*	74 i u	50 i u §
Chlortetracyclin	1st International Standard	1000 i u	1000 i u
Dihydro streptomycin	Internal Standard *compared to the* 1st International Standard	766 i u	1000 i u
Erythromycin	1st International Standard	950 i u	1000 i u †
Flavomycin	Farbwerke Hoechst AG	925 µg	100 µg in 50 per cent v/v of methanol
Lincomycin	The International Reference Preparation	881 i u	1000 i u
Neomycin sulphate	*Upjohn Company Ltd, Michigan, U S A*	675 µg Neomycin base	500 µg Neomycin base
Oleandomycin	*1st International Standard*	845 i u	1000 i u †
Oxytetracyclin	2nd International Standard	880 i u	1000 i u †
Penicillin G	Internal Standard compared to the 1st International Standard	1650 i u	1000 i u
Spiramycin	The International Reference Preparation	3200 i u	1000 i u †
Tylosin	1st International Standard	1000 i u	1000 i u †

* The International Standards and Reference Preparations are required at W H O International Laboratory for Biological Standards, National Institute for Medical Research, Mill Hill, London N W 7 England

§ Dissolve in 5 ml of 0 1 N HCl before further dilution with water

† *Dissolve in 5 ml of methanol before further dilution with water*

Electrolyte, pH 8 0 25 ml of the phosphate *buffer solution pH 8 0* were diluted to 1 litre with distilled water

Buffered agar solution A sterile 2 per cent w/v *solution of agar (Difco Bacto Agar)* was prepared After sterilization of 100 ml of the wanted phosphate buffer solution 2 5 ml of this was diluted to 100 ml with the agar solution previously melted by heating at 100° C The solution was kept in a water bath at 50° C until use At this temperature the solution may be kept for 30 hours

Bromophenol blue solution 100 mg of Bromo-

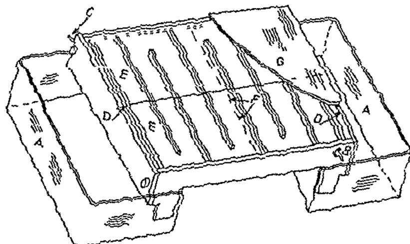


Fig 1 Apparatus for Electrophoresis A Electrolyte trays B and C Tap water inlet and outlet, D Hollow rooms for agar bridges E Cooling table with two gel containing glass plates F Application sites G Glass lid

phenol blue was dissolved in 100 ml of a 50 per cent v/v solution of ethanol in water

Reagents of analytical reagent grade were used when possible

Sterilizations were carried out by autoclaving (15 minutes at 120° C)

Preparation of Agar Gel Layers and Electrophoresis

The apparatus for electrophoresis and the technique for preparing agar layers used in this work was similar to that described by *Johansson* (3) except that the cooling surface of our apparatus was large enough to contain two 200 × 100 × 1 mm glass plates (see Fig 1) The equipment including the apparatus was washed in ethanol before use and by means of buffered agar solution (50° C) agar bridges connecting agar strips and agar gel layers were produced as described by *Johansson* (3) (A 1 mm thick U formed frame was placed between two glass plates of the dimensions given above) An electrolyte with a pH similar to that of the buffered agar solution was used Slits in the agar were then produced by pressing the long edges of 12 × 7 mm pieces of Whatman filterpaper no 4 vertically into the agar These were removed after 1 minute and into the slits 5 μl of freshly prepared antibiotic solutions (see Table 3) were applied 5 μl of bromophenol blue solution was applied into one slit This could give information of any possible irregularities during the course of the electrophoresis The plates were then transferred to the cooling table and the potential across the plates was adjusted to a value between 310 and 360 volts The current did not exceed 50 mA After 5 minutes the slits were covered with drops of melted buffered agar solution and the electrophoresis continued for a further 25 to 40 minutes

Bioautography

The two plates were then transferred to a bench and by means of a double bladed knife, two strips of agar (width 6 mm length approximately 180 mm) were cut out perpendicular to each of the slits For the detection of the separated components contained in the strips these were transferred to layers of agar seeded with various test organisms For each antibiotic a suitable test organism is suggested (see Tables 2 and 3) The preparation of the seeded agar layers is described below The microorganisms were then incubated over night at the temperatures given in Table 2 The positions of the antibiotics were now evident as zones of inhibition The nature of the zones (i e form, zone edges, the approximate size) were noted and the migration distance of each antibiotic (i e the distance between the application site and the centre of the spot) was determined

Preparation of Seeded Agar Layers

As we wanted to separate a mixture in which the antibiotics were present in low levels it was essential to test that the seeded layers to be used were sufficiently sensitive to each of the antibiotics Therefore, before any electrophoretical separation was run, a preliminary test of the seeded agar layers was carried out The purpose of this was to determine the volume of inoculum that should be used in the agar medium to give inhibition zones of a suitable size

Preliminary sensitivity test The microorganisms and the media used in the following are given in Table 2 The media were chosen as recommended in Federal Register (2) For *Bacillus stearothermophilus* the medium had the following composition

TABLE 2 *Solutions and Microorganisms used in the Preliminary Sensitivity Test*

Microorganism	Incubation temperature ° C	Difco* medium	Antibiotic	Concentration per ml	Diameter of zone mm
Sarcina lutea ATCC 9341	37	11	Erythromycin	0 08 i u	25
Micrococcus flavus ATCC 10240	37	1	Bacitracin	0 015 i u	13
Bacillus cereus var mycoides ATCC 11778	37	8	Chlortetracyclin	0 03 i u	15
Bacillus subtilis ATCC 6633	37	11	Erythromycin	0 03 i u	20
Staphylococcus epidermidis ATCC 12228	37	11	Neomycin base	1 μg	21
Bacillus stearothermophilus var calidolactis C 953 from L U F A Kiel Germany	50	Not§ Difco	Flavomycin	0 05 μg	26

§ The medium for the Flavomycin test is given under Material and Methods Incubation for 40 hours may be necessary for *Bacillus stearothermophilus*

* Bacto Antibiotic Media

TABLE 3 *Results Obtained by Identification*

Antibiotic	Concentration (per ml) applied	Seeded agar used (see Table 2)	Migration distance*, mm pH 8 0	pH 6 5	Diameter of Spots, mm	Edge of spots
Neomycin sulphate	50 μg of Neomycin base	E	–	22 +	12	distinct
Penicillin G	2 i u	A	–	11 +	10	diffuse
Flavomycin	0 25 μg	F	–	8 +	9	distinct
Dihydrostreptomycin	5 i u	D	–	1 +	8	diffuse
Chlortetracyclin	1 i u	C	–	19 –	15	diffuse
Oxytetracyclin	4 i u	C	–	20 –	11	diffuse
Bacitracin	1 5 i u	B	–	43 –	14	distinct (tailing)
Tylosin	5 i u	A	–	59 –	9	distinct
Spiramycin	5 i u	A	65 –	63 –	10	very distinct
Erythromycin	1 i u	A	71 –	66 –	8	diffuse
Oleandomycin	10 i u	A	72 –	63 –	8	distinct
Lincomycin	7 i u	A	–	74 –	10	very diffuse

* + and—means migration towards the anode and cathode, respectively

5 g of peptone, 4 g of yeast extract, 3 g of beef extract, 20 g of agar and 1 litre of distilled water Difco Bacto products were used and pH was adjusted to 7 0 after the sterilization by autoclaving Agar slope cultures of each microorganism were produced and inocula were prepared under strictly asceptic conditions by washing off the growth with a sterile solution of sodium chloride (0 9 per cent w/v in water) An aliquot of inoculum was thereafter added to a sterile melted agar medium at 50° C and the agar was poured into sterile petri dishes (diameter 13–30 cm) on a flat surface The thickness of the layer should be 1 7 mm and to facilitate an evenly distribution of the agar, plastic dishes were used Holes (diameter, 12 mm) were then punched in the agar and with a pipette 0 10 ml of the antibiotic solution was introduced into each hole The concentrations of the antibiotic solutions used are given in Table 2 The dishes were incubated over night at the temperatures given in Table 2 If the diameters of the inhibition zones formed were smaller than indicated in Table 2, then layers with less inoculum were prepared and the test procedure was repeated

TABLE 4 *Sensitivity of the Seeded Agar Layers to Each Antibiotic*

Antibiotic*	A *Sarcina lutea*§	B *Micrococcus flavus*	C *Bacillus cereus*	D *Bacillus subtilis*	E *Staphylococcus epidermidis*	F *Bacillus stearoth*
Bacitracin	—	+	—	—	—	—
Chlortetracyclin	—	(+)	+	(+)	—	—
Dihydrostreptomycin	—	—	—	+	—	—
Erythromycin	+	—	—	—	—	—
Flavomycin	—	—	—	—	—	+
Lincomycin	+	—	—	—	—	—
Neomycin sulphate	(+)	(+)	—	(+)	+	—
Oleandomycin	+	—	—	—	—	—
Penicillin G	+	—	—	(+)	—	—
Spiramycin	+	—	—	—	—	—
Tylosin	+	+	—	—	—	—

* The concentrations of the antibiotics are given in Table 3
§ The seeded agar layers used have the sensitivity given in Table 2
\+ means zones of the sizes given in Table 3 (+) and — means very weak zones and no zones, respectively

until the diameters given in Table 2 were obtained

Seeded agar layers for identification New plates were then prepared as described above with agar containing the above determined amount of inoculum The plates were stored in a refrigerator and were used within a few hours The various inocula however could be stored in a refrigerator for two to four weeks

RESULTS AND DISCUSSION

The present identification of the antibiotics is based upon three principles, i e the different migration distances the varying sensitivity of the microorganisms used and the edge and form of the spots

Experiments showed that the diameter of the spots, obtained after electrophoresis and development of the separated components on the seeded agar plates described in Table 2 ranged from 8 to 15 mm Table 3 shows that it was possible to separate and identify 9 antibiotics simultaneously i e Neomycin sulphate Penicillin G Dihydrostreptomycin Chlortetracyclin, Bacitracin Tylosin Spiramycin and Lincomycin By replacing Chlortetracyclin with Oxytetracyclin in the mixture it appeared that it was not possible to differentiate between these two antibiotics

Table 4 shows that most of the test organisms used were sensitive to more than one antibiotic Another feature which may also help in the identification was the edge of the spots (Table 3) but it should be mentioned that if the zones obtained were smaller than those indicated in Table 3 all edges were diffuse

If Spiramycin Oleandomycin and Erythromycin were all present in a mixture the identification was not possible when the electrophoresis was carried out at pH 6 5 However at pH 8 0 the spot of Spiramycin could be distinguished from the spot indicating the mixture of Oleandomycin and Erythromycin The difference of the zone edges of Oleandomycin and Erythromycin should be noted since this may help in the identification of these in samples in which only one of them is present

In our opinion the result of the present identification procedure is frequently of little value unless it is accompanied by a rough indication of the concentration of the substance under investigation Especially if zones of an antibiotic which is expected to be present are found to be absent, this result is seldom interesting unless information as to the sensitivity of the method is given Therefore, a test of the sensitivity of the seeded agar layers (Table 2) was included in the method

Results obtained by methods for quantitative assay of small amounts of an antibiotic will often be affected by the presence of other antibiotics In such cases a semiquantitative determination by the present method may give better information

Although some of the tested antibiotics contain more than one derivative (e g Bacitracin Flavomycin Neomycin), we have only noticed one spot for each of the antibiotics under investigation The reasons for this may be the following That the different forms are migrating at identical velocities, that only one form is present in sufficiently high concentration to give any antibacterial effect and finally that only one form has any biological activity

The method reported here should be particularly useful in the identification of antibiotics present in for example tissue body fluids or animal feed stuffs It has the advantage of being easily extended to antibiotics other than those described in this work Since up to 16 different microorganisms may be used after each electrophoretic separation, many antibiotics may be identified simultaneously

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AGGLUTININS AGAINST GROUP III ATYPICAL MYCOBACTERIA

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The epidemiological significance of agglutinins against Group III atypical mycobacteria was studied by testing a panel of sera with nine different *M intracellulare* antigens Five of them represented "common" types most frequently isolated from clinical specimens, the other four represented "rare" types never isolated from such specimens Agglutinins were detected in human sera more frequently against the "common" types than against the "rare" types The greatest proportion of reactive sera and the highest titres were found with the serotype Chance antigen In bovine and porcine sera there was no difference in the occurrence of antibodies against the "common" and "rare" serotypes, and the serotype Chance antigen did not exhibit any exceptional reactivity The observations are compatible with the view that the antibodies in human sera to a considerable extent result from immunization by the mycobacterium type used for serological testing or a closely related type

Skin testing with tuberculin and related sensitins has been widely used in epidemiological studies for the detection of persons infected by mycobacteria (4) Corresponding studies with humoral antibodies have not been carried out

In contrast to *M tuberculosis*, stable suspensions for agglutination tests can be made of many atypical mycobacteria Advantage has been taken of this in developing a system for their serological classification (5) Many human sera contain low-titred agglutinins against atypical mycobacteria Although they are hardly of diagnostic significance, their investigation may be useful for epidemiological purposes The present communication was designed to shed light on this question

MATERIAL AND METHODS

The following strains provided by Dr *W Schaefer* from the National Jewish Hospital at Denver were used

type IIIa	strain Iowa
type IIIb	strain 14186–1424
type IV	strains P55 and 13528–1079
type V	strains 4443–1237 and 25546–759
type VI	strains 34540 and 12315/67
type VII	strains P49 and Manten 157
type Howell	strains 4549 and P42
type Chance	strains Chance and Lynn H
type Altman	strains Melnick and 2219
type Yandle	strains Yandle and ATCC15987
type Davis	strains SG B 2 and 14658–1686
type Boone	strains P39 and T-2970

Bacteria were cultivated and suspensions for agglutination tests prepared as described by *Schaefer* (5)

Unless specifically stated, the two antigens representing each serotype were combined to make the final test antigen For the agglutination reaction, 0 2 ml volumes of progressive twofold dilutions of the serum were mixed with equal volumes of the bacterial suspension The reading was made after 5 hours' incubation at 37° C Titres were expressed as reciprocals of dilutions, calculated from the total volume of reagents in the tube

Normal human sera were taken from pregnant women and sent to the laboratory for routine syphilis screening Sera from miscellaneous hospital

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TABLE 1 *Occurrence of Mycobacterial Agglutinins in Human Sera*

Test antigen	Healthy persons 54 cases No with titre		Hospital patients 100 cases No with titre		Tuberculous patients 34 cases No with titre		Total 188 humans per cent with titre
	≧ 16	≧ 64	≧ 16	≧ 64	≧ 16	≧ 64	≧ 16
III	1	0	4	0	1	1	3
V	1	0	0	0	1	0	1
VI	0	0	2	0	0	0	1
Altman	1	0	2	0	1	0	2
IV	2	0	7	0	1	0	5
Chance	40	10	67	16	19	4	67
Yandle	0	0	5	0	1	0	3
Davis	11	1	12	0	11	0	18
Boone	7	1	11	1	2	0	11

patients were picked up among specimens sent to the laboratory for routine syphilis screening

Specimens from patients with verified (18 sera) or suspected (21 sera) tuberculosis were collected primarily for another purpose in connection with bronchoscopy The series did not include patients with far advanced tuberculosis Bovine and porcine sera were mainly taken from animals with respiratory or gastrointestinal complaints, and the specimens were sent to the laboratory for virus serological studies

RESULTS

Four of the antigens used in the study represented "rare" types, i e types not isolated from clinical specimens in Finland (*M intracellulare* serotypes III, V, VI, and Altman)

Five antigens represented "common" types, i e types most frequently isolated from clinical specimens (*M intracellulare* serotypes IV, Chance, Yandle, Davis and Boone)

Table 1 shows the occurrence of agglutinins in 188 human sera against these antigens Antibodies were detected more frequently against the "common" types than against the "rare" types They occurred most commonly by far against the serotype Chance and the average titre of reactive sera against this antigen was also higher than corresponding titres against other antigens However, in 10 of 62 sera with no agglutinins against the serotype Chance they were found against some other antigen The agglutinin titre in human sera rarely exceeded 64 and in no instance was it over 128

TABLE 2 *Occurrence of Mycobacterial Agglutinins in Animal Sera*

Test antigen	Swines 20 cases No with titre		Cattle 33 cases No with titre		Total 53 animals per cent with titre
	≧ 16	≧ 64	≧ 16	≧ 64	≧ 16
III	10	5	30	12	76
V	8	2	20	1	53
VI	99	2	31	3	76
Altman	1	0	18	1	36
IV	4	2	22	1	49
Chance	9	4	30	9	74
Yandle	6	1	26	2	60
Davis	10	7	33	5	81
Boone	4	1	20	2	44

No essential difference in the occurrence of antibodies was observed between healthy persons miscellaneous hospital patients and patients with verified or suspected tuberculosis Thirty human sera were tested with four different strains representing serotypes VIII and Howell which are antigenically closely related to serotype Chance The two strains of type VII and one of the two strains of type Howell yielded results very similar to those obtained with the Chance antigen The other Howell strains yielded only a few lowtitred reactions Thus the behaviour of this strain resembled the behaviour of the other serotypes used for testing

Results obtained with animal sera are shown in Table 2 The over all occurrence of antibodies was much higher than in human sera and there was no difference between the common and rare serotypes The highest titre recorded was 256 i e of the same order of magnitude as in human sera Antibodies against the serotype Chance were detected at about the same frequency as against other types Finally antibodies were detected at a somewhat higher frequency in bovine than in porcine sera

DISCUSSION

Much effort has been made to develop a reliable serological test for the diagnosis of tuberculosis Numerous techniques have been devised but they all have failed to differentiate clearly between tuberculous and nontuberculous subjects The same holds true for skin testing used as an indicator of cellular hypersensitivity against mycobacterial antigens moderate reactivity does not exclude active tuberculosis and on the other hand such reactivity is frequently found in persons without clinically manifest tuberculosis

When sensitive radioimmunological techniques are used humoral antibodies can be detected in most if not all human sera (2) Immunogens involved may be derived from saprophytic mycobacteria (2)

The purpose of the present communication was to obtain information on the eventual epidemiological significance of agglutinins against Group III atypical mycobacteria The experience gained in the preparation of antisera in rabbits for typing purposes indicates that the agglutinin response against these bacteria is fairly specific i e the titre with homologous antiserum is usually 2–4 twofold dilution steps higher than titres with other serotypes (1 5) Antisera for typing are usually not absorbed although the absorption may be necessary with closely related serotypes (3)

Selection of the test strains was based on information on the occurrence of Group III mycobacterial serotypes in Finland (3) All these types are very rare as compared with *M tuberculosis* since 75 strains belonging Group III were isolated from sputum specimens sent to the tuberculosis department of the Central Public Health Laboratory during a ten year period and associated with disease with some confidence Agglutinins in human sera against the test antigens obviously can result from immunization by either a) the mycobacterium type used for serological testing or a closely related type b) some other mycobacterium species or type that shares antigenic determinants group antigens with the test strain or c) completely unrelated but crossreacting material

By far the highest number of reactive human sera was found with the serotype Chance antigen The reason for this may be either that there is indeed more antibody combining with this type or that this type is more readily agglutinable than other strains With animal sera the serotype Chance anti

other antigens used for testing Patients with tuberculosis obviously do have more antibodies against *M tuberculosis* than healthy controls No difference was observed in the present series between healthy controls and patients with verified or suspected tuberculosis hence the immunogen involved may not be derived predominantly from *M tuberculosis*

Antibodies were detected in human sera more frequently against the "common" types than against the "rare" types A corresponding difference was not observed in animal sera These findings favour the view that the antibodies in human sera, at least to a considerable extent, result from immunization by the mycobacterium type used for serological testing or a closely related type This would mean that specific contact with many atypical mycobacteria giving rise to antibody response is a very common occurrence indeed

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THE EFFECT OF ANTIBIOTICS ON THE GROWTH AND LONGEVITY OF HUMAN DIPLOID FIBROBLASTS *IN VITRO*

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The influence of various mixtures of 18 antibiotics on the growth rate and longevity of human embryonic diploid lung fibroblasts was investigated A basic mixture of 100 IU/ml penicillin, 100 ug/ml streptomycin, 10 IU/ml mycostatin and 1 μg/ml fungazone was established and other antibiotics were added to this, mostly at a concentration of 10 μg/ml The antibiotics were incorporated in the medium and the cells were grown constantly in their presence The following antibiotics added to the basic mixture increased longevity significantly and in some cases even increased the over all growth rate above the culture containing only penicillin and streptomycin kanamycin, geramycin, aureomycin, lincocin, aureomycin + fugacillin, aureomycin + humycin, and aureomycin + erythromycin Some antibiotics added to the basic mixture caused cell degeneration after 25 to 40 days *in vitro* geramycin + humycin, geramycin + terramycin, aureomycin + achromycin, and aureomycin + geramycin + chloramphenicol Some others produced lesser inhibitions terramycin, geramycin + fugacillin, geramycin + erythromycin, and geramycin + kanamycin Many mixtures gave diploid cell growth rates and longevities equal to or greater than the control Such mixtures may be useful in controlling microbial contamination occurring in cell cultures

Microbial contamination is a constant threat to the cultivation of all mammalian cells *in vitro* and particularly human embryonic diploid lung fibroblasts (HEDLF) grown in serial passage Contamination results in a great economic loss in the production of human viral vaccines as well as a loss of time and effort in research The penicillin and streptomycin used in most media are inadequate to protect against many gram negative microorganisms and molds Other antibiotics are occasionally used but there has been no systematic study of the effect of various antibiotics and combinations of antibiotics on the growth and longevity of human diploid cells

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biotics is used which may have a synergistic effect against microorganisms and permit the use of lower concentrations of these substances The present report describes a systematic survey of the effect of 18 antibiotics in various combinations on the growth and longevity of human diploid fibroblasts

MATERIAL AND METHODS

Cells HEDLF strains were grown from aborted human embryonic lung tissue in this laboratory Two independent strains of cells were used for

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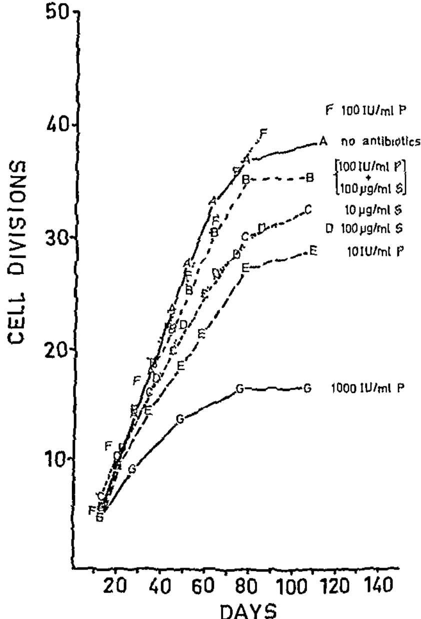


Fig 1 Tl e growth response of HEDLF cells grown in medi m without antibiotics or co a n ng e tl er 10 100 or 1000 IU/*ml Penicillin* (P) 10 100 ug/ml Streptomycin (S) or a mixture f 100 IU/ml P + 100 µg/ml S 1000 µg/ml S was toxic

these experiments with essentially ident cal results Tl e growth characteristics of these cells are s m lar to *those of* the *HEDLF* cells described by *Hayflick* & *Moorhead* (2) and *Hayflick* (3)

Medium Eagle s minim m essential medium (MEM) (1) was u ed in all experiments supple mented with *10* per cent calf serum 4 mM L glutamine 1 mM Na pyruvate and sufficient Na bicarbonate to make tl e initial pH of the medium about 7 0

Culture tecl nique Tl e general tecl n ques for c lt vating and passing the cells l ave been de scribed elsewhere (4 6 7 8) Tl e cells v ere grown in Jena glass culture bottles w tl a growth sur face of 46 cm² The bottles were noculated with 2×10^5 cells in a volume of 20 m l med um When confl ent the cells were trypsi zed wit 0 25 per cent tryps n in Hanks l uffer pH 8 0 at room temperature Tl e suspended cells w re centrifuged at 1 000 × g for 15 minutes at 4 C resuspended

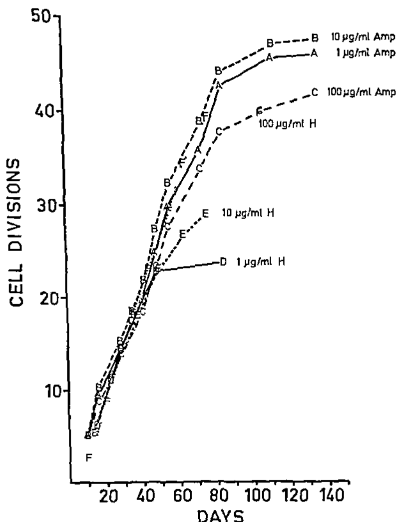


Fig 2 The growth response of HEDLF cells grown in medium containing either 1 10 or 100 µg/ml Ampicillin (Amp) or 1 10 or 100 µg/ml Humycin (H) The 10 µg/ml H culture became contaminated just as the cells were becoming senescent

in 5 ml of the growth medium in which they were accustomed to grow and counted with a haemocytometer To minimize variations of growth due to inconsistent dishwashing the same bottle was re used without intermittent cleaning at each passage This procedure was found to have no influence on growth rate or logevity if compared to the usual procedure of adding cells to washed bottles at each passage

When cells approached senescence the time at which the cell population ceases to grow they were maintained for 1 month with a change of medium once a week If the culture did not become confluent within this time the cells were trypsinized counted and discarded or if too few the number of cells on the glass surface was counted directly with an inverted microscope by the same procedure used to measure the proportion of attached cells (6 7)

The proportion of inoculated cells attaching to the glass was measured the day after they were seeded by a procedure described earlier (6 7) These values were used to determine the efficiency of attachment with each passage and to calculate the number of cell divisions

Antibiotics The antibiotics used were purchased as sterile solutions or powders and dissolved in distilled water to a concentration of 1 or 5 mg/ml These stock solutions were stored according to the manufacturer s recommendation at 4° C or 20° C One hundred ml medium with antibiotics were

made-up at any given time, stored at 4° C and used until exhausted, then new media were made

The following antibiotics were used

Achromycin (Ach)	Lederle, USA
Ampicillin (Amp)	Kabi, Sweden
Aureomycin (A)	Lederle USA
Chloromphenicol (C)	Apothekernas Lab, Norway
Colistinat (Col)	Lundbeck, Sweden
Erythromycin (E)	Lilly, USA
Fugacillin (Fug)	Astra Sweden
Fungazone (F)	Squibb, USA
Geramycin (G)	Shering, USA
Humycin (H)	Parke Davis USA
Kanamycin	Ferrosan Denmark
Lincocin (L)	Upjohn, USA
Mycostatin (M)	Squibb, USA
Neomycin (N)	Upjohn, USA
Penicillin, benzyl (P)	Kabi Sweden
Polymyxin B (Poly)	Pfizer, USA
Streptomycin (S)	Kabi, Sweden
Terramycin (T)	Pfizer, USA

The abbreviations are used in the figures and tables

RESULTS

Each of the above was first studied individually as the sole antibiotic in the medium at concentrations of 1, 10 and 100 μg or IU per ml Only one out of four control cultures started without antibiotics did not become contaminated The growth response of this cultures as well as a culture containing 100 IU/ml penicillin + 100 μg/ml streptomycin (P,S) is given in Fig 1 The effect of different concentrations of penicillin or streptomycin present individually in the medium are also shown One thousand IU/ml penicillin was highly inhibitory and 1000 μg/ml streptomycin was toxic to cells Although 10 μg/ml and 100 μg/ml streptomycin gave identical results, 10 IU/ml penicillin yielded much poorer growth than 100 IU/ml This effect of a lower growth response at lower concentrations of a substance which is not an essential nutrient was observed also with humycin (Fig 2), colistinat (1 IU/ml gave slightly lower longevity than 10 IU/ml) and aureomycin (1 μg/ml gave poorer growth than 10 μg/ml, data not shown)

At 100 μg/ml ampicillin, a broad ranged penicillin, similar growth results were obtained as 100 IU/ml penicillin and better longevity than the mixture penicillin + streptomycin, but at 10 and 1 μg/ml ampicillin gave much better growth than corresponding concentrations of penicillin (fig 2)

Polymyxin B showed almost equal inhibitory effects over a concentration range of 1 to 100 IU/ml (Fig 3) One IU/ml gave slightly poorer growth than either 10 or 100 IU/ml

Geramycin showed increasing inhibitory effects with concentration (Fig 3) However, at 10 ug/ml the growth rate was not affected for the first 22 cell divisions after which the cells rapidly became senescent

Ten ug/ml erythromycin, chloramphenicol and terramycin, and 10 IU/ml colistinat all gave an equal or better growth response than the control without antibiotics and the culture containing penicillin + streptomycin (Fig 4)

The growth results obtained by the other antibiotics used singly were either equal to or slightly less than the controls at 1 and 10 μg/ml or were interfered with by contamination

In the author's laboratory the most common contaminants encountered were yeasts or other fungi Therefore a mixture consisting of 100 IU/ml penicillin 100 μg/ml streptomycin, 10 IU/ml mycostatin and 1 μg/ml fungazone was used Henceforth in this paper, this combination shall be referred to as the basic mixture In cases of overt infection mycostatin can be increased to 100 IU/ml and fungazone to 10 μg/ml for at least one month with little inhibitory effects on diploid cell growth The other antibiotics were added to this basic mixture at a concentration of 10 μg/ml or IU/ml except for achromycin which was used at 1 μg/ml

The results illustrated in Figure 5 and listed in Tables 1 and 2 show the variations in growth rate longevity and average cell attachment obtained with the different antibiotic combinations In spite of the presence of a large number of antibiotics many cultures

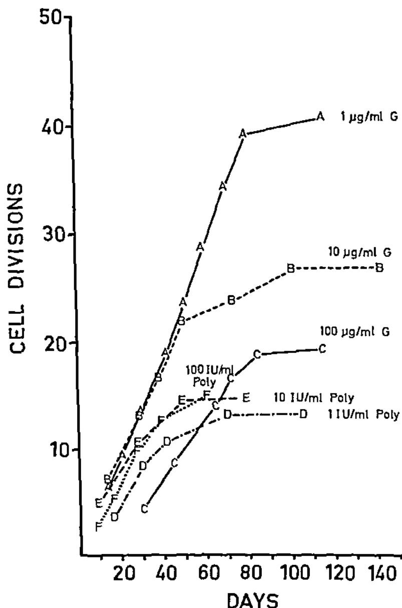


Fig 3 The growth response of HEDLF cells grown in medium containing either 1, 10 or 100 μg/ml Geramycin (G), or 1, 10 or 100 IU/ml Polymyxin B (Poly)

eventually became contaminated towards the terminal stage of their growth by some form(s) of bacterial agent (Table 1) No effort was made to identify the microorganism(s) The times at which all of the cultures showed this contamination were very similar suggesting a single exposure The penicillin + streptomycin and basic mixture cultures, contaminated after about 65 cell divisions, each appeared to be very close to senescence In a second experiment (Table 2), no contamination occurred

From Tables 1 and 2 and Fig 5 it is apparent that a number of antibiotics added to the basic mixture increased the longevity of the cell population significantly aureomycin,

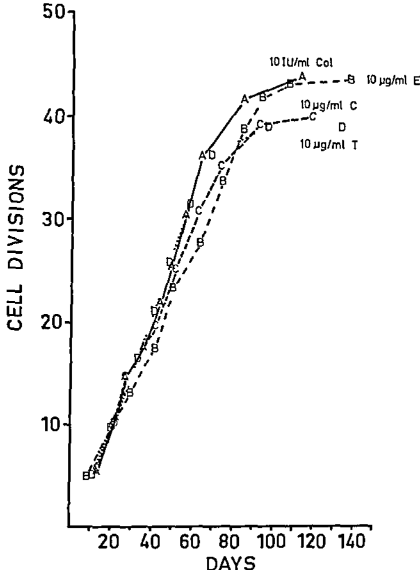


Fig 4 The growth response of HEDLF cells grown in medium containing either 10 IU/ml Colistinat (Col) 10 µg/ml Erythromycin (E) 10 µg/ml Chloromphenicol (C) or 10 µg/ml Terramycin (T) At 100 µg/ml or IU/ml these antibiotics were either inhibitory or toxic

geramycin kanamycin aureomycin + fugacillin aureomycin + erythromycin aureomycin + humycin aureomycin + lincocin Some antibiotics added to the basic mixture stimulated an increased growth rate aureomycin aureomycin + erythromycin, aureomycin + fugacillin aureomycin + humycin aureomycin + kanamycin aureomycin + lincocin aureomycin + neomycin aureomycin + polymyxin B aureomycin + geramycin + fugacillin aureomycin + geramycin + erythromycin aureomycin + geramycin + kanamycin These results represent the only observations in the author s laboratory of conditions which produced an increase in the overall growth rate of HEDLF cells above that of the control

In addition to stimulatory effects some mixtures had a markedly toxic effect on the cells which caused degeneration of all cells after 25 to 50 days *in vitro* basic mixture plus aureomycin + achromycin, geramycin

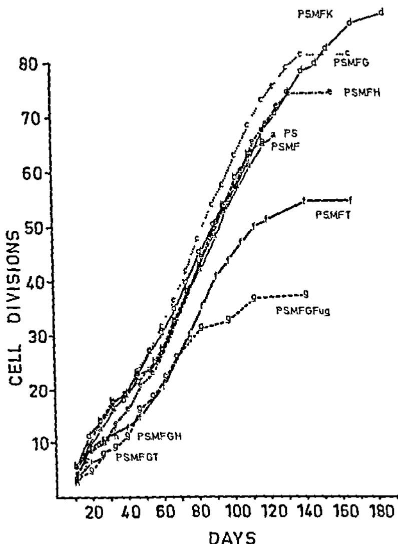


Fig 5 The growth response of H biotics Penicillin + Streptomyc S M,F), P,S,M,F + Kanamycin + Terramycin (T) P S M,F + Geramycin + Terramycin The following concentrations were used 100 IU/ml Penicillin, 100 μg/ml Streptomycin 10 IU/ml Mycostatin 1 μg/ml Fungazone and 10 μg/ml Geramycin, Humycin, Fugacillin, Kanamycin and Terramycin

+ humycin, geramycin + terramycin aureomycin + geramycin + chloramphenicol although aureomycin achromycin geramycin humycin, aureomycin + geramycin aureomycin + chloramphenicol and geramycin + chloramphenicol gave fair to excellent growth when added to the basic mixture The basic mixture plus geramycin + fugacillin was inhibitory to a lesser extent Some of these mixtures reduced the growth rate as well as shortened the life expectancy, however, cells exposed to the basic mixture plus geramycin + erythromycin had the same growth rate as the control but ageing was accelerated after 45 cell divisions Very subtle effects were also apparent, for example, although the basic

TABLE 1 *The Effect of Various Mixtures of Antibiotics on the Growth, Ageing and Average Cell Attachment of HEDLF Cells*

Antibiotic mixture*	Total no cell div	Cell div at senesc	Time senesc occurred (days)	Cell div † / days	Total time days	Ave % cells attached
P,S	66			0 54	123§	66
P,S,M,F	65			0 56	117§	61
P,S,M,F,K	88	87	166	0 53	202	64
P,S,M,F,G	81	81	139	0 58	165	66
P,S,M,F,A	65			0 60	109§	76
P,S,M,F,Col	58			0 57	102§	63
P,S,M,F,Amp	51			0 50	102§	80
P,S,M F,N	72	71	131	0 54	165	68
P,S,M,F,Poly	67			0 57	117§	72
P,S,M,F,Ach	56			0 55	102§	74
P,S M,F,L	64			0 59	109§	71
P,S,M,F,E	75	73	131	0 56	154	72
P,S M,F,H	75	75	131	0 57	154	64
P,S,M,F,C	59			0 54	109§	54
P,S M,F,Fug	60			0 59	102§	78
P,S,M,F,T	55	50	111	0 45	165	53
P,S,M,F,G,K	56	51	97	0 53	147	63
P,S,M,F,G,N	62	58	104	0 56	165	58
P,S,M,F,G,L	64	58	111	0 52	182	69
P,S M,F,G,A	61	60	131	0 46	182	82
P,S,M,F,G Col	63	62	111	0 56	147	68
P,S,M F,G,E	54	50	97	0 52	140	70
P S,M,F,G,Fug	37	31	82	0 38	140	66
P,S M,F,G,C	60	56	124	0 45	182	61
P,S M,F,G H	11			0 33	33$	29
P,S,M,F,G,T	8			0 31	26$	36

* The antibiotics were present at the following concentrations 100 IU/ml penicillin (P), 100 µg/ml streptomycin (S), 10 IU/ml mycostatin (M), 1 µg/ml fugazone (F), 10 µg/ml geramycin (G), ampicillin (Amp), kanamycin (K), aureomycin (A), fugacillin (Fug), erythromycin (E), neomycin (N), chloramphenicol (C), terramycin (T), lincocin (L), humycin (H), 10 IU/ml polymyxin B (Poly), colistinat (Col) and 1 µg/ml achromycin (Ach)

† Calculated by dividing cell divisions at senescence by time senescence occurred, or in the case of contamination total no cell divisions by total *in vitro* time

§ Culture contaminated

$ Cells degenerated

mixture plus aureomycin + geramycin yielded fair growth compared to the control, the growth rate was reduced and senescence accelerated significantly was compared to cultures grown in the presence of the basic mixture plus either aureomycin or geramycin

In general at the concentrations used most of the antibiotic mixtures permitted growth patterns similar to that of the culure containing penicillin streptomycin Thus, there are a number of antibiotic mixtures that could be used in growth medium over long periods of time which would either improve the growth response of HEDLF cells or give growth comparable to the control

Most of the antibiotics gave average cell attachment values which were similar to the attachment in the penicillin + streptomycin culture The range of values usually varied from 100 per cent to as low as 10 per cent of the cells in the inoculum attaching Those mixtures which were inhibitory to growth gave poor attachment also

In the production of viral vaccines, peni-

TABLE 2 *The Effect of Various Mixtures of Antibiotics on the Growth Ageing and Average Cell Attachment of HEDLF Cells The Antibiotic Concentrations Are the Same as Those in Table 1*

Antibiotic mixture	Total no cell div	Cell div at senesc	Time senesc occurred (days)	Cell div * / days	Total time days	Ave % cells attached
P S	52	51	92	0 55	147	76
P S M F,A	68	68	115	0 59	143	71
P S M,F A Fug	70	62	91	0 69	147	61
P S M F,A E	61	60	92	0 65	147	72
P S M F,A,K	59	56	92	0 61	147	72
P S M F,A Poly	57	43	71	0 61	147	75
P,S M F A N	59	48	78	0 62	147	79
P S M F,A C	52	46	92	0 50	147	51
P S M,F A Ach	20	16	29	0 55	58†	61
P S M,F A T	55	53	87	0 61	147	62
P S M F A L	64	63	100	0 63	107	68
P S M F A H	65	59	92	0 64	147	65
P S M F A Col	55	51	85	0 60	147	66
P S M F A G Fug	50	47	71	0 66	147	68
P S M F,A G E	55	52	79	0 66	147	62
P S M F A G K	59	50	78	0 64	147	63
P S M F,A G C	18	18	35	0 52	43†	53

* Calculated by dividing cell divisions at senescence by time senescence occurred
† Cells degenerated

cillin is usually omitted from the medium and streptomycin used alone or together with neomycin Therefore it was of some practical interest to investigate the effect of these two antibiotics on HEDLF cells when used together at various concentrations The results listed in Table 3 show that streptomycin alone can be used over a concentration range of 100 to 500 μg/ml with similar growth responses and attachment values Neomycin alone in the medium at 10 and 100 μg/ml gave poorer growth than the penicillin and streptomycin control and poorer attachment with increasing concentration

If neomycin at 10 μg/ml was mixed with streptomycin at 100 and 200 μg/ml there was a slight stimulatory effect on the growth rate but at 500 μg/ml streptomycin the growth rate and longevity was decreased

With the different concentrations of streptomycin after one passage with 50 μg/ml neomycin and during the first passage with 100 μg/ml neomycin a marked change in the morphology of the cell monolayer occurred

Large masses of cells could be seen to accumulate at discrete spots on the surface partly due to retraction of cells from the glass in that area These cell masses eventually sloughed off the glass and floated in the medium leaving large holes in the remaining cell layer This tendency was not so marked if 100 μg/ml neomycin was used alone Some attempt was made to collect the clumps floating in the medium at the time the culture was trypsinized and to estimate the cells but most of these cells sl[illegible] degeneration Therefore, the [illegible] of those cultures containing 50 and 100 μg/ml neomycin plus [illegible] less than the true amount of cell [illegible] 3) The cell attachment was also [illegible] in cultures containing 50 and 100 [illegible]mycin with the various [illegible] streptomycin After about 4 [illegible] in the continual presence of these [illegible] about 95 per cent of the cells in the [illegible] did not attach to the glass.

TABLE 3 *The Effect of Various Concentrations of Streptomycin (S) and Neomycin (N) on the Growth, Ageing and Average Cell Attachment of HEDLF Cells*

Antibiotics	Total no cell div	Cell div at senesc	Time senesc occurred (days)	Cell div * / days	Total time days	Ave % cells attached
100 IU/ml P + 100 µg/ml S	38	35	83	0.41	124	84
10 µg/ml N	27	26	64	0.41	72†	70
100 µg/ml N	27	27	84	0.32	114	51
100 µg/ml S	36	36	90	0.40	120	84
200 µg/ml S	36	36	90	0.40	120	61
500 µg/ml S	35	33	83	0.40	124	79
100 µg/ml S + 10 µg/ml N	35	35	83	0.42	120	68
200 µg/ml S + 10 µg/ml N	39	39	90	0.43	120	59
500 µg/ml S + 10 µg/ml N	23	22	57	0.39	84	48
100 µg/ml S + 50 µg/ml N	27	26	40	0.65	77	49
200 µg/ml S + 50 µg/ml N	29	29	57	0.51	84	52
500 µg/ml S + 50 µg/ml N	13			0.59	22†	49
100 µg/ml S + 100 µg/ml N	21	18	40	0.45	77	55
200 µg/ml S + 100 µg/ml N	16	16	57	0.28	84	44
500 µg/ml S + 100 µg/ml N	14	13	40	0.33	77	53

* Calculated by dividing cell divisions at senescence by time senescence occurred, or in the case of contamination total no cell divisions by total *in vitro* time

† Culture contaminated

DISCUSSION

Contamination has been a constant problem in the author's laboratory The fact that most of our antibiotic-free cultures usually became contaminated within a few weeks suggests that our cell cultures are repeatedly exposed to this possibility It is believed that air-borne microorganisms can only partly explain the contamination observed In our laboratory a bottle can be left open on an exposed table for several minutes before the chances of contamination would become serious A frequent source appears to come from "sterile" components such as calf serum trypsin, buffers and other solutions added to the medium Large rubber stoppers have been found to contain viable spores after 3 hours autoclaving and even sterile disposable filter units used to sterilize some of the medium components have been found contaminated The standards used to determine sterility may not be sensitive enough to detect microorganisms at the very low concentrations which may eventually contaminate cultures under conditions used in our laboratories, for example, contaminations occur most frequently in older culture which have almost reached senescence These cells have been exposed to a large volume of the same batch of medium In addition, when contamination occurs it usually involves a large number of independent cultures and not just a sporadic few This problem becomes particularly acute when large volumes of medium are used for large-scale cell cultivation

Since it is very difficult to control the sterili-

ty of all the components used in cell cultivation the initial purpose of this investigation was to establish complex mixtures of antibiotics which would give a broad range of protection against microbial contamination and not interfere with the growth of human diploid cells Although we have not performed experiments to determine the effectiveness of protection against various microorganisms, the information has been applied successfully in a special field of dental research to control microbial contamination on extracted teeth incubated *in vitro* for studies on tooth replantation and transplantation (10, 12) In some cases overt infections with molds have been cured by applying some of the antibiotic mixtures and increasing the mycostatin concentration to 100 IU/ml and the fungazone to 10 μg/ml

Since all antibiotics are metabolic inhibitors, it is reasonable to assume that even those which have no apparent immediate effect on cells may have a subtle effect which would become obvious over long exposures Previous experience has suggested that older cells are more sensitive to inhibitory chemicals or conditions than younger ones (9) Since cell ageing is a very sensitive indicator of inhibitory or stimulatory substances, such subtle effects may become apparent with diploid cells which might be overlooked in cell lines which do not age such as HeLa cells

If individual antibiotics were used (partly shown in Figures 1 4) the data obtained demonstrated that the growth response of HEDLF is similar whether grown without antibiotics or in the presence of 100 IU/ml penicillin + 100 μg/ml streptomycin (Fig 1) However, 100 IU/ml penicillin only gave a better growth response than 100 μg/ml streptomycin and the penicillin + streptomycin curve appears to be a resultant of the two effects whenever the antibiotics are mixed implying that there are no synergistic or antagonistic interactions between these two substances

The unusual growth response between 10 and 100 IU/ml penicillin (Fig 1) 10 and 100 μg/ml humycin (Fig 2) 1 and 10 IU/ml colistinat (data not shown) and 1 and 10 μg/ml aureomycin (data not shown) where the HEDLF longevity was reduced as the antibiotic concentration was lowered cannot be explained at present This type of relationship was also observed if DL-ethionine was added to the medium at concentrations of 1 μg/ml and lower (unpublished results) A great deal more will have to be learned about the mechanism of cell ageing before the significance of these observations can be evaluated

It is apparent from Fig 2 that 100 μg/ml ampicillin, a broad ranged penicillin, produced a similar growth response as 100 IU/ml penicillin and a better growth response than 100 IU/ml penicillin + 100 μg/ml streptomycin Fugacillin, another broad ranged penicillin was much more growth inhibitory at 100 μg/ml if used alone than ampicillin or benzyl penicillin at this concentration Since these antibiotics differ at only one point in their structure, it may be possible to use such antibiotics in studies on the chemical groups which affect growth and cell ageing

If used alone the effect of some antibiotics on cell growth would change markedly when they were added to the basic mixture For example, 10 μg/ml geramycin alone in the medium was inhibitory to HEDLF cells (Fig 3), although earlier results (5) as well as the results listed in Table 1 and Fig 5 show that at this concentration together with penicillin and streptomycin geramycin produced a marked increase in longevity In addition, the growth inhibitory effects of 10 IU/ml polymyxin B was completely neutralized and 10 μg/ml humycin produced a marked increase in longevity Terramycin on the other hand, reduced the growth rate and longevity when present in this mixture, whereas it permitted good growth when present alone in the medium (Fig 4) At the present time it has not been determined which antibiotic(s) in the basic mixture interact with the antibiotics mentioned above to produce these effects on growth and longevity

The results show that geramycin, aureomycin, kanamycin and to a lesser extent

erythromycin and humycin increased longevity significantly when added to the basic mixture at a concentration of 10 μg/ml In addition, some of the mixtures studied gave a greater over all growth rate than the control These data support the suggestion that the greater longevity and growth rates produced by the various antibiotic mixtures may result from direct action of these substances on the cells by interfering with some of the growth regulating and perhaps ageing mechanisms However, because of the relatively high concentrations of antibiotics used in our media, the specificity of their action may be low and several biological mechanisms may be involved Of course, a possible repeated exposure of these cells to low levels of contamination by various agents may play a role in their growth response This possibility may be studied by deliberate infection of these cells with microorganisms susceptible to the various antibiotics used

The observation that some antibiotic mixtures (geramycin + humycin, geramycin + terramycin geramycin + fugicillin aureomycin + achromycin or aureomycin + geramycin + chloramphenicol) produced a highly inhibitory effect on cell growth was unexpected because each one of these antibiotics when added separately to the basic mixture gave good or excellent growth Antagonistic and synergistic interactions between different groups of antibiotics have been described by *Manten* & *Wisse* (11) but these groups did not produce similar effects in our system However these authors were comparing the action of antibiotics against microorganisms It is possible that the mechanisms of action which produced the stimulatory or inhibitory effects in HEDLF cells was not the same as those which influence bacterial cells What we call antibiotic antagonism the production of an increased toxicity against diploid cells or synergism the production of an increased growth rate or longevity might have a different or no effect from a bacterial reference point

As long as the regulations on the use of antibiotics in medium used to prepare viral vaccines are so restrictive, it is not possible to apply some of the growth stimulating antibiotic mixtures for this purpose The results recorded in Table 3 suggest that the mixture of 200 μg/ml streptomycin plus 10 μg/ml neomycin gave the best growth results However, to avoid large loses of cells during the passage procedure due to poor attachment it may be advisable to inoculate cells into new cultures in medium free of antibiotics and then add the antibiotics the following day after the cells had a chance to attach For possible better protection against microbial contamination one could increase the neomycin concentration to 50 μg/ml and the streptomycin concentration to 500 μg/ml but for no longer than 2 passages or 2 weeks *in vitro*

The results presented in this paper indicate that it is not possible to predict from the growth response to individual antibiotics the effect to be obtained if they are mixed in various concentrations Furthermore since the relationship between the results with *in vitro* grown human diploid cells and animal experimentation have yet to be studied it is difficult to evaluate their significance for human application However the possibility exists that the growth studies with HEDLF cells described in this paper may eventually be useful in tests of the acceptability or toxicity of mixtures of antibiotics or other pharmacologically active substances The possibility that some antibiotic mixtures may have antiviral activity or even anticancer effects should be investigated

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ROLE OF BLOCKING SERUM FACTORS IN ORGAN GRAFTED RATS

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The aim of the present study was to investigate whether inhibition of the mixed lympho cyte culture (M L C) interaction by sera from organ transplanted rats could be used as an indicator of the development of immunological enhancement The findings may be summarized as follows 1) Conventionally produced immune sera exhibited an antigen specific inhibition of M L C whether directed against the stimulating or the responding cells 2) Sera from organ transplanted rats also consistently inhibited the M L C interac tion However, no difference was found between sera from rats who had rejected their grafts and from recipients of long term surviving grafts 3) Sera from long term survivors and post rejection sera proved equally capable of inducing passive enhancement *in vivo*

The development of immunological enhancement depends on the action of antibodies in the recipient against the grafted tissue (8) In recent years, enhancement regimes have been shown to be active in prolongating the functional survival times of allogeneic kidney (11, 16) and heart (6, 7) transplants in rats The reason for allograft acceptance in these experiments is thought to be due to an immunoblocking effect of serum antibodies or antigen antibody complexes on the graft or on the cellular immune system (1, 9)

Since the mixed lymphocyte culture (M L C) response is known to be an *in vitro* counterpart to the inductive phase of the allograft reaction *in vivo* we felt interested in examining what effect the addition of serum from allografted rats would have on the M L C response between lymphocytes from normal recipient strain and donor strain rats The main questions that we wanted to answer were 1) How would conventionally raised imune sera influence M L C responses, and could any allogeneic specificity be seen 2) How would serum from rats who had rejected allografts function compared to 3) the effect of serum from "enhanced" long term acceptors

METHODS AND MATERIAL

Inbred rats of the following strains and their F1 hybrids were used

Wistar (W1), Fischer (F1) AS AS2 and Brown Norway (BN)

The F1 and AS strains are compatible at the major histocompatibility locus and carry the H 1[l] (= AgB[1]) allele

The W1 strain carry the H 1[w] (AgB[4]), the AS2 strain the H 1[f] and the BN strain the H 1[n] (= AgB[3]) alleles The different donor recipient combinations and M L C responder stimulator combinations used in the present study were in all experiments performed across the major histocompatibility barrier

Rats aged from 4 8 months of both sexes were used in the experiments

Cervical heart transplantation in the rat was used as the grafting system in that this system had proved convenient for enhancement studies in earlier work (4, 6) In brief, a vascularized heart transplant was placed subcutaneously in the lateral

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triangle of the neck and the function followed by daily inspections and/or palpation of the beats The end point of rejection used was total loss of electrical activity on ECG traced over the graft

The regimen for production of active enhancement in the rat recipient was the following Donor strain bone marrow cells washed twice in tissue culture medium was counted and adjusted to contain from 0 7 2 0 × 10⁷ viable mononucleated cells *per ml medium One ml of this solution was injected intravenously in the tailvein of the prospective recipient* and transplantation was carried out, using donor strain heart of same genotype as the bone marrow cells, after a period of from 7 20 days later A group of recipients of same sex and age were heart grafted without having received any pretreatment

Except for anaesthesia and the administration of 150 000 i u penicillin i m postoperatively no drugs were given

During a period of approximately 4 weeks, two samples of serum for passive enhancement experiments were taken each week from the tails of individual W_1 rats Serum was frozen and stored at −20° C until the serum samples from each rat were pooled separately In this way five pools of serum were obtained from W_1 rats who had rejected $(W_1xBN)F1$ heart allografts, and five pools of serum *from W_1 rats carrying long* term accepted $(W_1xBN)F1$ heart allografts

These pools of sera were tested for passive enhancing properties in normal W_1 recipients grafted with $(W_1xBN)F1$ heart allografts and compared with the effect of serum pools taken from normal non-immune Wi rats 1 ml of serum was injected intraperitoneally on the day of transplantation, and *1 ml on day 2, day 3, and every other day until a* total of 10 ml had been injected in recipients given the most massive serum treatment Some sera were tested in doses of from 0 5-1 ml and, if so, the injection was given only on the day of transplantation Total volumes greater than 1 and less than 10 ml were administered according to the time schemes outlined above, but the injections were stopped when the desired volume had been given

"Conventionally" immune sera were raised by monthly injections of $2\ 0 \times 10^7$ viable bone marrow cells intravenously Sera were pooled from several rats of same sex and strain, which had all received same immunization

The M L C technique used was as previously described (14, 15) In brief, blood obtained by heart puncture was defibrinated and sedimentated with dextran The leucocytes were washed twice

TABLE 1 *Survival Times of Heart Allografts in the Different Donor Recipient Combinations*

		Heart transplant removed or rejected completely at indicated day
$(W_1 \times BN) \rightarrow W_1$	Normal	6–13*
	Bone Marrow Pretreated	20, 22, 33, 328†, 390†, 394†, 405†, 510† 525†, 544
$F_1 \rightarrow$AS2	Normal	8, 8, 10, 10 10, 10, 10, 13, 14, 16
	Bone Marrow Pretreated	43†, 45†, 55, 140†, 150 , 150 , 270
AS × BN → AS × AS2	Normal	8, 9, 9, 10, 10, 11, 14, 16, 16, 17, 18, 18, 20, 31, 35, 42, 240†
	Bone Marrow Pretreated	6, 8 13, 14, 18, 19, 19 20, 22, 60
AS2 × BN → AS × BN	Normal	7, 8, 9, 10, 10, 10, 10, 11, 11, 13, 13, 15, 20
	Bone Marrow Pretreated	7, 7, 7, 16, 16, 16, 17, 17, 17, 18, 19, 19, 24, 62, 78, 330†
AS × BN → AS2 × BN	Normal	12, 14, 29, 152, 158 230, 240 , 240§, 270§, 270§, 300§
	Bone Marrow Pretreated	8, 8, 9, 11, 12, 13, 27, 40, 210§, 240

† Killed while the heart transplant was well functioning
§ Still functioning graft
* Range, 20 recipients (Ref No 5)

TABLE 2 *(W1×BN)F1 Heart Allografts in W1 Rats Treated with Serum (Passive Enhancement) Survival Times in Days*

Serum volume injected	Serum originating from: Non immune W1 rats (control group)	W1 rats who rejected W1×BN hearts	W1 rats with accepted W1×BN hearts
0 5 1 0 ml	8, 8 9	12, 150†, 182†	
1 0– 5 0 ml	5, 10	9, 60, 186†, 245†	10, 12, 14, 16, 129†, 320†
5 0–10 0 ml	6, 8, 8, 8, 8, 11	20, 163†, 360†	12, 16, 18, 21§, 358§ 440
		$p<0\ 01$	$p<0\ 01$

The passive transfer schedule is described in Methods and Materials
† Rats killed while heart grafts were well functioning
§ Heart grafts still functioning

and finally resuspended in Eagles medium containing 5 per cent fresh heat inactivated rat serum Culture volumes were 0 5 ml containing $0\ 2\times10^{6}$ responding and $0\ 2\times10^{6}$ mitomycin treated stimulating cells The proliferating activity was assessed by the incorporation of ^{14}C thymidine during the last 16 hours of the 96 hours' culture period

The test for immunoblocking effect of serum was performed by the addition of the heat inactivated recipient serum (or recipient serum diluted in normal serum) in a final concentration of 5 per cent to the cultures The exact set up will be seen from the presentation of the results

Serum was obtained from rats by cardiac puncture In most M L C inhibition experiments freshly drawn immune serum was used while a few experiments were performed with sera that had been frozen and stored at −20° C for a few months As no detectable differences between sera treated in either way have been seen no further distinction shall be made and no comments on this will be given in the results

RESULTS

The results of the transplantation experiments are summarized in Table 1 The outcome of heart grafting in the W1×BN→W1 and in the F1 → AS2 combination is parellel to that obtained in other similar studies using parental strain rat recipients (6, 7, 11) namely acute rejection in the non pretreated recipients and prolongation of graft survival (active enhancement in the bone marrow pretreated group

In the (AS BN F1→(AS × AS2)F1 combination, norm l rats rejected within less sharply limited p ds and one rat exhibited spontaneous long term acceptance In this combination, however, the standard enhancement pretreatment regimen had no significant effect on graft survival (Wilcoxon test)

Normal (AS×BN)F1 recipients rejected (AS2×BN)F1 hearts within 7-20 days, and bone marrow pretreatment had moderate, but significant effect in prolonging graft survival ($p=0\ 02$ Wilcoxon rank sum test), but only few long-term acceptors were obtained

In the (AS × BN)F1→(AS2 × BN)F1 combination spontaneous graft acceptance was found in the majority (75 per cent) of normal recipients, whereas bone marrow preimmunization in this combination, as a whole, resulted in acceleration of graft rejection ($p<0\ 01$, Wilcoxon)

Passive Enhancement Experiments

Pooled serum taken from four individual rats who had rejected heart allografts and from four rats carrying well established grafts was investigated for its ability to prolong heart graft rejection in non immune rats The pooled results of this type of experiment which was performed in the W1×BN → W1 combination are shown in Table 2 It was found that the sera in either group as a whole had significantly enhancing properties compared to sera from non immune normal rats

M.L C Inhibition Experiments

It has been reported in studies of man (2 12), rats (3), and dogs (10) that serum-

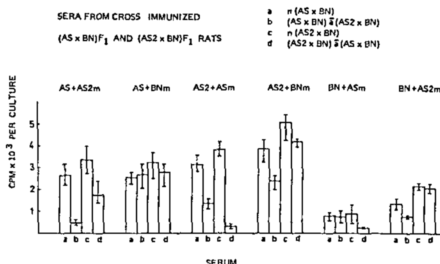


Fig 1 Shows Exp 2 in Table 3 Columns and vertical bars indicate median and range of the quadruplicate determinations

mediated inhibition of M L C failed to show strict antigen specificity in that the stimulating and responding capacity of third party cells (different from the actual donor and recipient employed in the immunization) was diminished as well, although to a less degree than that of the donor cells

This could be due to antigenic determinants not known to be shared by the donor and the third party cells In order to exclude this possibility, experiments were made with sera produced in F1 hybrids thus excluding the possibility of antibodies directed against histocompatibility antigens of either parental strain

The experimental scheme appears from Fig 1 and the results of all four experiments performed are listed in Table 3 One-way M L C's were prepared in all possible combinations of AS, AS2, and BN cells To these mixtures were added a AS2 serum prepared in (AS×BN)F1 hybrids and a AS serum prepared in (AS2 × BN)F1 hybrids together with the corresponding control sera

It is seen that the antisera regularly inhibited the M L C's if directed against either the responding or the stimulating cells Furthermore a clear-cut antigen specificity of the inhibition was obtained Non specific inhibition was seen only in one out of 16 cases (Exp No 3, a AS serum in the AS2+BNm mixture)

Sera from the heart grafted rats were tested for MLC inhibiting capacity in cultures containing the relevant donor antigens on the stimulating cells (specific MLC's), in cultures lacking these antigens (control MLC's), and in unstimulated cultures In most donor-recipient groups, serum from individual rats who had rejected acutely (i e within 20 days after grafting) and serum from rats who turned out to be long term acceptors (i e longer than 42 days after the grafting) was investigated

The sera were taken after varying periods in the interval between 18 80 days after transplantation, and four long term survivors in the F1 → AS2 group were bled 150 days after transplantation

The results appear from Table 4 and two typical experiments are shown in Fig 2 It is seen that the recipient sera in all cases inhibited the "specific MLC s" whereas only very few 'control MLC s" were inhibited,

TABLE 3 Serum mediated Inhibition of MLC Sera from Cross immunized (AS × BN) F1 and (AS2 × BN)F1 Rats

XTURE	SERUM†	EXP NO 1 CPM§	2 CPM	3 CPM	4 CPM
+ AS2*m*	a	4342 (3966–5091)	2626 (2197–3157)	2568 (2208–3240)	5407 (4723 5933
	b	1308 (1208–1662)	484 (380– 639)	1269 (1163–1396)	1373 (815 1818)
	c	5767 (5585–5967)	3370 (2731–3999)	2703 (2370–3263)	5085 (4757–6025)
	d	3354 (2670–3853)	1766 (1416–2431)	879 (755– 973)	4336 (3728–5286)
+ BNm	a	4911 (4448–5449)	2512 (2276–2804)	3242 (2991–3470)	4464 (4242–5462)
	b	5587 (4964–5855)	2693 (2060–3174)	3586 (3053–4493)	4579 (4160–4877)
	c	6364 (6343–7086)	3248 (2512–3690)	3467 (3065–4112)	4943 (3417–5445)
	d	6030 (4862–6409)	2808 (2136–3187)	2201 (1638–2600)	4334 (4098–4833)
) + ASm	a	1892 (1649–2207)	3152 (2825–3587)	3538 (2988–3870)	4676 (4305 5009)
	b	1197 (1197–1198)	1384 (1111–1585)	1843 (1739–1906)	1764 (1489 2937)
	c	2781 (2601–3537)	3842 (3536–4208)	4119 (3924–4472)	4375 (4218 4926)
	d	1096 (857–1125)	311 (215– 433)	305 (231– 426)	805 (639 934)
) + BNm	a	5185 (5144–5688)	3853 (3263–4282)	4499 (3471–4868)	5904 (5120–7052)
	b	1588 (1539 2247)	2396 (2014 2649)	2669 (2463 2884)	3433 (2657–3708)
	c	5698 (1589–5892)	5090 (4252–5426)	5205 (4918–5680)	5422 (5308–6913)
	d	4841 (4207–5968)	4175 (3951–4287)	3803 (3691–3878)	5916 (4932–7756)
+ AS*m*	a	1077 (777 1117)	792 (745 958)	1159 (816–1185)	1332 (1060–1674)
	b	1235 (986 1303)	740 (480–1025)	985 (893–1039)	1343 (808–1656)
	c	1521 (1231–1634)	879 (459–1303)	1411 (1241–1617)	1439 (1152–1927)
	d	828 (780– 941)	267 (221– 328)	618 (380– 770)	882 (694–1166)
+ AS2m	a	2014 (1798–2103)	1340 (1083 1601)	1062 (1046–1088)	1504 (1148–1850)
	b	1009 (980 1038)	764 (690– 823)	802 (588– 955)	948 (671–1276)
	c	2363 (2354–2372)	2154 (1990–2283)	1157 (1109–1168)	1723 (1433–1952)
	d	2074 (1833–2157)	2069 (1863 2245)	963 (810 1211)	1585 (1357–2000)

† a n(AS × BN), (/ ... BN) a (AS × BN)
§ CPM per culture
The serum-pools test ... the same groups of rats 2 6 months after initiation of the immunizations

thus confirming the antigen specificity of the serum factors involved

No recipient sera influenced the low activity in the unmixed control cultures

The MLC inhibition system however, totally failed to distinguish between the action of sera from recipients who had rejected and from recipients showing prolonged allograft acceptance

The presence of complement fixing lymphocytotoxic antibodies in low titres were characteristic for sera of either group and these low titres are in contrast to the high dilutions in which these sera did exhibit specific M L C blocking activity

The way in which serum mediates inhibition of M L C is not known To exclude any cytotoxic action as the main reason a previous report from this laboratory (15), has shown that the response to P H A added to the cultures in optimal doses was not inhibited by recipient sera directed against the responding cells In these experiments the antisera were added to the cultures simultaneously with P H A and the possibility existed that the P H A in some way protected

TABLE 4 *Serum mediated Inhibition of MLC Sera from Heart Graft Recipients*

Donor - recipient combination	Fate of graft†	Inhibition§ of specific MLC*				Control MLC$			
		a	b	c	d	a	b	c	dþ
1 (W1×BN) → W1	R	+	+	+		+	—	—	
	R	+	+	—		+	—	—	
	R	+	—	—		—	—	—	
	R	+	+	—		—	—	—	
	R	+	—	—		—	—	—	
	S	+	+	—		—	+	—	
	S	+	—	—		—	—	—	
	S	+	+	—		—	—	—	
	S	+	+	—		—	—		
	S	+	+			—	—	—	
2 F1 → AS2	S	+				—			
	S	+				—			
	S	+				—			
	S	+				—			
3 (AS×BN) → (AS2×AS)	R	+	+	+		—	—	—	
	R	+	—	—		—	—	—	
	R	+	+	—		—	—	—	
	S	+	+	—		+	—	—	
	S	+	+	—		+	—	—	
	S	+	+	—		—	—	—	
4 (AS2×BN) → (AS×BN)	R	+	+	—	-	—	—	—	—
	R	+	—	—	—	—	—	—	—
	R	+	+	—	-	—	—	—	—
	R	+	+	+		+	+	—	
	R	+	+	+		—	—	—	
	R	+	+	—	—	—	—	—	—
	R	+	+	+	+	—	—	—	—
	S	+	—	—	—	—	—	—	—
5 (AS×BN) → (AS2×BN)	R	+	+	+	—	—	—	—	—
	R	+	—	—	—	—	—	—	—
	R	+	+	—	—	—	—	—	—
	R	+	+	—	—	+	—	—	—
	S	+	+	—	—	—	—	—	—
	S	+	+	—	—	—	—	—	—
	S	+	+	+	—	—	—	—	—
	S								

† S Long term surviving R Rejected

§ Inhibition was considered positive if no overlap occurred between the quadruplicate determinations of cultures containing control serum and of cultures containing recipient serum

* Cell partners in specific MLC's Group 1 W1 + (W1×BN), 2 AS2 + (AS2×F1) 3 AS2 + (AS2×BN), 4 AS + (AS2×AS) 5 AS2 + (AS2×AS)

$ Cell partners in control MLC's Group 1 W1 + (W1×F1), 2 AS2 + (AS2×BN), 3 AS2 + (AS2×AS), 4 AS + (AS×BN) 5 AS2 + (AS2×BN)

þ Final dilution of recipient serum in the cultures a, b, c, d, is 1/20, 1/200, 1/2000, and 1/20000 respectively

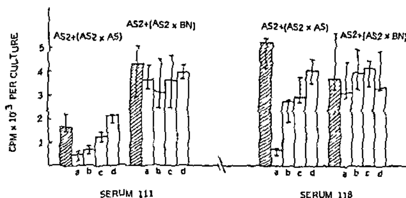


Fig 2 Test of two recipient-sera from Group 5 (see Table 4) The heart graft in recipient no 111 was still functioning 300 days after the transplantation The serum was collected 53 days after the operation Recipient no 118 rejected the graft after 12 days The serum was collected 12 days later Columns and vertical bars indicate median and range of the quadruplicate determinations Cross-hatched columns indicate MLC's with control (AS2×BN)F1 serum Columns a, b, c, and d show activity after culture with recipient serum in the final dilutions 1/20, 1/200, 1/2000, and 1/20000, respectively.

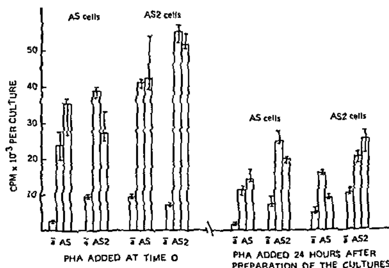


Fig 3 Shows one typical experiment out of a total of four experiments performed Columns and vertical bars indicate median and range of the quadruplicate determinations Each group of three columns show the results of stimulation with 0 25 µl 1 00 µl, and 4 00 µl of PHA per ml of culture All cultures were harvested 2 days after the addition of PHA

the cells from a cytotoxic action by the antisera Therefore, in the present study experiments have been performed where the addition of P H A was delayed 24 hours after the cells had been mixed with the antisera in culture It is seen from Fig 3 that this procedure failed to reveal any inhibition by the specific antisera of the P H A response and

this was true both after addition of optimal and of suboptimal doses of PHA

DISCUSSION

The results obtained in the present investigations were unexpected in several respects

The reason why more than one histoincompatible donor recipient combination was used for the grafting experiments was to be able to draw more generally valid conclusions It had been expected that acute rejection of the heart allografts would take place in all combinations in the non immunized rats, and that the pretreatment with donor cells would condition the recipients for long term acceptance of the graft, as demonstrated in a number of other inbred rat combinations (6, 7, 11, 16) The expectation was fulfilled in the combination of $W_1 \times BN \rightarrow W_1$, $F_1 \rightarrow AS2$, and, partly, in $AS2 \times BN \rightarrow AS \times BN$, but certainly not in $AS \times BN \rightarrow AS \times AS2$, or in $AS \times BN \rightarrow AS_2 \times BN$ where a number of spontaneous graft acceptors developed, and where bone marrow pretreatment was either without effect or had the contrary effect of accelerated rejection of the subsequent heart transplant The high percentage of spontaneous acceptors in the last combination agrees to a certain extent with the spontaneous accept of kidneys trans planted from AS to AS2 rats shown earlier (13 15) A regular and convincing produc tion of enhancement in the two other com binations where F1 hybrid recipients were employed has also proved difficult in later experiments where passive enhancement schedules were employed, the explanation of this failure remains obscure The results in general accentuate that caution must be exercised before generalizations are made on the bases of studies of the production of enhancement in a single rat strain combination

For the sake of clarity, sera from heart grafted rats tested in the MLC inhibition studies originated from typical recipients which had rejected acutely, whether or not they were bone marrow pretreated, and from typical recipients who had well functioning hearts which continued beating for long periods after, whether these rats had accepted spontaneously or following pretreatment It has been shown earlier that this kind of graft acceptance, whether induced spontaneously (15) or following pre-immunization (6) is attributable to active enhancement states and not to immunological tolerance

The MLC technique detected specific blocking factors in serum, but the inhibition was similar in serum after rejection and during prolonged graft survival Furthermore, no difference in cytotoxicity between the two kinds of sera was found

Several explanations of these results may be offered However, the finding that also post-rejection serum had *in vito* enhancing properties, similar to serum from long time graft acceptors, indicates that immuno-regulatory antibodies may be produced in re sponse to all allografts The fate of the graft may thus depend mainly on the strength and the time-course of the cell mediated immune response provoked by the graft

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IMMUNE RESPONSE TO *MYCOBACTERIUM LEPRAE* IN INDETERMINATE LEPROSY PATIENTS

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mune responsiveness to *Mycobacterium leprae* was examined in thirtyone histologically ssified indeterminate leprosy patients Fourteen of the patients were also classified as eterminate clinically (strictly indeterminate group), while the other seventeen patients re clinically classified as tuberculoid or borderline leprosy The strictly indeterminate oup appeared to be quite homogenous in their immune reactivity to *M leprae* All patients vealed a lymphocyte transformation of less than 3 per cent (mean 0 57 ± 0 88) and nly 1 out of 7 patients tested by the leucocyte migration technique revealed a migration idex of less than 0 80 (mean 0 91 ± 0 16) Only one patient gave a positive early lepromin eaction None of the patients revealed a positive reaction in gel precipitation to mycobacterial antigens These findings are in agreement with the view that the immune response to *M leprae* has not been triggered off in strictly indeterminate leprosy On the other hand, the clinically tuberculoid and borderline patients with an indeterminate histological picture responded on average more strongly to *M leprae*, and by and large according to their clinical diagnosis It is concluded that in classifying patients as indeterminate leprosy, the clinical picture may give more information than histopathology

In many leprosy patients the first sign of disease is one or a few vague hypopigmented macules At this early stage the lesions usually lack sufficient definite clinical and histological characteristics to be placed accurately in the leprosy spectrum ** This condition is called indeterminate leprosy The indeterminate macule(s) may regress spontaneously or progress to become tuberculoid, borderline or lepromatous leprosy As a result of regression the histological picture may revert to indeterminate, but this is less likely to be case clinically

As immunity to *Mycobacterium leprae* is cell mediated (18) it seems reasonable to assume that the varied further course of indeterminate leprosy reflects the strength of the cell-mediated immune response mounted against the infecting organism A close corre-

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** Leprosy patients, except the indeterminate patients may be grouped into a spectrum according to immunity ranging from the high resistant polar tuberculoid (TT) type through the borderline groups (BT, BB BL) to the low resistant, progressive polar lepromatous (LL) type (12 15)

lation has been found between the clinico histopathological picture throughout the leprosy spectrum and the immune responsiveness to *M leprae* as evaluated by the methods of lymphocyte transformation leucocyte migration inhibition and delayed skin hypersensitivity (12) On the other hand, in highly bacilliferous patients where cell mediated immunity to *M leprae* is low or absent, precipitating humoral antibodies may be detected against soluble mycobacterial antigens (see 18)

In the present study, these methods have been used to assess the status of immunity in patients with a histopathological diagnosis of indeterminate leprosy

MATERIALS AND METHODS

Patients

The patients were attending the out patient clinic at All Africa Leprosy & Rehabilitation Training Centre (ALERT)

Clinical diagnoses were based on clinical examinations and skin smear bacterial indices (13) A skin biopsy was taken from all patients and histological classification done blindly without clinical information

Indeterminate leprosy was clinically defined as a condition consisting of one or a few hypopigmented macules usually with some sensory loss but with normal peripheral nerves (1) Patients with other clinical manifestations of leprosy were grouped according to *Ridley & Jopling* classification (15) The histological picture was considered to be indeterminate when there was no granuloma present but one or more of the following features were seen 1 infiltration of lymphocytes and histocytes around skin appendages peripheral nerves and vessels with or without proliferation of spindle shaped cells in the superficial dermis 2) proliferation of Schwann cells or 3) acid fast bacilli were found in nerve arrector pili muscles or sub epidermal zone (14)

Fourteen patients were both clinically and histologically classified as indeterminate Their age ranged from 8 to 38 years and their length of history of disease from 2 months to 5 years Eleven of the patients were untreated and three had received one dosage of 4 4 diamino-diphenyl sulfone (DDS) prior to the immunological investigations

Seventeen patients clinically classified as tuberculoid or borderline showed the histological features of indeterminate leprosy Most of them claimed that they had not received any anti leprosy therapy when admitted to the study

Antigens

M leprae was obtained from skin biopsies from patients with lepromatous leprosy (5) Lepromin was prepared according to the WHO standard (20) BCG (Glaxo) was used for the lymphocyte transformation test For the gel diffusion analysis BCG and *Mycobacterium duvalii* (17) were cultured on Sauton medium and disintegrated by X press and ultrasonic treatment (11)

Sera

Sera were obtained from the same blood as used for the lymphocyte transformation test and stored at 20° C until tested

Lymphocyte Transformation

The method is fully described in a previous paper (5) The transformation was routinely quantitated morphologically and expressed as percentage transformed cells For confirmation of the morphological reading the incorporation of tritiated thymidine was also measured in a few cases and the radioactive uptake expressed as test/control (T/C) ratio (6)

Leucocyte Migration Inhibition

The technique used is described elsewhere (6) Results were expressed as

$$\text{Migration index} = \frac{\text{Average area of migration with antigen}}{\text{Average area of migration without antigen}}$$

Skin Testing

The method of lepromin testing has been described in a previous paper (12) and reactions recorded as recommended by WHO (19)

Gel Diffusion

Double diffusion in gel analyses were performed in 1 per cent agarose (8) The test was carried out as direct precipitation reactions between patient sera and antigens released from disintegrated BCG and *M duvalii* bacilli (11)

RESULTS

Immune Response in Patients Classified both Clinically and Histologically as Indeterminate Leprosy (Strictly Indeterminate Leprosy)

The immune response to *M leprae in vitro* and *in vivo* is shown in Table 1 The blastoid response was in all patients negative or low (mean percentage 0 57 ± 0 88) being less

TABLE 1 *Immune Response to M leprae in Patients Classified both Clinically and Histologically as Indeterminate Leprosy (Strictly Indeterminate Leprosy)*

Pat ent no	Lymphocyte transformation per cent	Migration index	Leprom n early	Leprom n late	Lymphocyte transformation after leprominteting per cent
26/71	<0 5				
32/71	<0 5		—	+	
155/71	0 5				
237/71	<0 5	1 14	—	+	
253/71	<0 5	0 96	—	+ + +	27 5
337/71	0 75		—	+	<0 5
353/71	<0 5	0 86	—	+—	2 0
99/72	0 5	0 88	+ +	+ + +	
125/72	<0 5	0 93			
202/72	<0 5	0 90			
K20/72	1 5		—	+	2 0
K48/72	2 5				
320/72	<0 5		—	+	<0 5
K107/72	2 25	0 73		+ + +	
Mean	*0 57 ± 0 88*	*0 91 ± 0 16*			

than 0 5 per cent in eight out of fourteen patients while the highest value recorded was 2 5 per cent The three patients who showed a blastoid response of more than 1 0 per cent all had only a single lesion The strength of response did not seem to be related to the length of history of disease Nor was the response to *M leprae* correlated to the response to BCG (correlation coefficient = 0 15), which varied considerably being 0 5 per cent in five and >10 per cent in three patients (mean 11 3 per cent)

Of the seven patients exam ned in the leucocyte migration inhibition test one showed significant inhibition of migration (patient no K 107/73) This patient also had a positive blastoid response 2 25 per cent) in contrast to the six patients without significant inhibition who were all negative in the transformation test

The early lepromin reaction was negative in all except one of the eight patients examined while the late reaction varied from + – to + + + In five individuals the transformation to *M leprae* was re examined after reading of the late lepromin reaction The results are shown to the right in Table 1 One patient, who had a + + + late reaction showed an increase in blastoid response from 0 5 per cent initially to 27 5 per cent after lepromin testing No clinical change was observed

Sera from ten patients were examined for precipitating anti mycobacterial antibodies using antigens released from disintegrated BCG and *M duvalii* bacilli All sera were negative

Immune Response in Patients Classified Clinically as Tuberculoid or Borderline and Histologically as Indeterminate Leprosy

The blastoid response to *M leprae* in this group of patients showed great variation (Table 2) The mean response was 4 6 per cent, more than seven times higher than in the other patient group The strength of the response tended to correlate with the clinical diagnosis being in average 9 3 per cent in the clinically TT patients 2 6 per cent in BT patients and 0 33 per cent in the clinically BL group A very good agreement was observed between the cellular incorporation of ^{3}H

TABLE 2 *Immune Response to* M leprae *in Patients Classified Clinically as Tuberculoid or Borderline and Histologically as Indeterminate Leprosy*

Patient no	Clinical diagnosis	Lymphocyte transformation			Migration		Lepromin	
		per cent	mean	T/C ratio	index	mean	early	late
82/70	TT	23 0						
109/70	TT	8 0		8 6				
110/70	TT	1 0	9 3 ± 8 5	2 3				
114/70	TT	0 5						
117/70	TT	13 5						
8/71	TT	10 0						
73/70	BT	2 0						
76/70	BT	9 5						
91/70	BT	2 5						
182/71	BT	0 5			0 90		—	+
286/71	BT	0 75	2 6 ± 3 3		0 98		+ +	+ +
60/72	BT	5 5			0 80	0 91 ± 0 18	+	
35/72	BT	<0 5		0 53	1 01		—	+ +
K49/72	BT	<0 5			0 84			
208/71	BL	1 0		1 4	0 90		—	—
236/71	BL	<0 5	0 33 ± 0 59		1 00	0 96 ± 0 14	—	—
289/71	BL	<0 5			0 97			

thymidine and the morphological transformation in the cultures from the four patients examined by both methods

The lymphocyte transformation with BCG as antigen also showed great variation The average response was 10 4 per cent, while the mean values for three clinical groups were TT 7 6 per cent, BT 9 1 per cent and BL 11 6 per cent Thus there was no correlation between the response to *M leprae* and BCG (correlation coefficient = 0 10)

Only clinically BT and BL patients were studied in the migration test (Table 2) One patient (60/72) showed a *M leprae*—induced inhibition of migration at the level of significance (0 80) The mean migration index was lower for the BT group (0 91) than for the BL group (0 96)

The early and the late lepromin reaction were examined in six and five patients respectively The clinically BL patients were negative in both the early and the late reaction while all the BT patients were positive in the late but showed a varied pattern in the early reaction This is in accordance with findings in histologically BT and BL patients (12)

Three sera (82/70, 91/70, 208/71) gave precipitating lines against BCG as well as *M duvalii* antigens One serum (208/71) from a clinically BL patient, gave two lines the other sera one line only Serum precipitins were found in one patient with a strong (23 0 per cent) and in one patient with a weak (2 5 per cent) lymphocyte transformation to *M leprae* indicating that a positive response in one test does not exclude a positive response in the other

DISCUSSION

The proportion of strictly indeterminate lesions that regress spontaneously apparently remains unknown *Lara* & *Nolasco* (10) in their extensive study on childhood leprosy found that in 37 per cent of children with flat macular lesions, the lesions disappeared within 3 years, but about 50 per cent of these lesions showed tuberculoid pathology It is

interesting to note that a higher proportion of lesions with clinical signs of tuberculoid leprosy with inflammation healed spontaneously (*Lara & Nolasco* 1956) In our experience these are the cases with the strongest immune responses to *M leprae* as measured by lymphocyte transformation and leucocyte migration inhibition (7)

Information seems also to be lacking concerning the proportion of indeterminate lesions which will progress into tuberculoid, borderline or lepromatous leprosy, although it is generally agreed by experienced leprologists that such movements may take place (1, 3, 9) Thus it is apparent that the natural history of indeterminate leprosy may vary from one patient to the other

However this varying potential of resistance was not detectable by *in vitro* methods which assess the cell mediated immunity to *M leprae* at the time when patients presented with indeterminate leprosy In our study only 2 out of 14 patients revealed a lymphocyte transformation to *M leprae* stronger than 2 per cent and one a leucocyte migration inhibition of less than 0 80 Thus it does not appear that the immunological *in vitro* investigations can provide valuable information regarding the prognosis of strictly indeterminate leprosy

On the other hand a more variable response was found in the strictly indeterminate group by the late lepromin reaction in which one third of the patients tested responded strongly This test has been found to be of prognostic value in indeterminate (4) and childhood leprosy (10) and apparently reflects differences in potential ability to eliminate *M leprae* by immune mechanisms

The negative lymphocyte transformation and leucocyte migration inhibition results indicate that in most patients with strictly indeterminate leprosy the immune apparatus has not yet started to respond to *M leprae* to such an extent that these factors can be measured in the peripheral blood The many negative early lepromin reactions and the lack of detectable humoral anti mycobacterial antibody support this conclusion These findings are in agreement with the view that the immune response to *M leprae* has not yet been triggered off in these patients (21), although a state of weak responsiveness expressed at a local level (in the lesion) only, may well be present

In one patient, with a strong late lepromin reaction, the lymphocyte transformation changed from 0 5 before to 27 5 after lepromin testing This indicates that the lepromin test itself may switch on the immune response to *M leprae* and may explain why repeated lepromin testing may enhance spontaneous recovery in childhood leprosy (10)

The clinically tuberculoid and borderline groups of patients showed roughly an immune response to *M leprae* in accordance with their place in the clinical spectrum. The response of the TT and BT groups was somewhat lower than that found when the immunological responsiveness of clinically and histologically typically cases was determined (12) This could partly be because we intentionally defined indeterminate rather strictly clinically, possibly classifying as TT or BT some patients with macules and neural manifestations that according to the resolution of the Madrid Congress (16) would be indeterminate

When re examining the slides after being informed of the clinical classification the pathologist could not histologically differentiate between the clinical indeterminate group and the group consisting of clinically tuberculoid and borderline patients In established or classifiable leprosy an alteration in the immunological situation is detected histologically before it is apparent clinically (7), and it is therefore surprising that in early or indeterminate leprosy the reverse should appear to hold true It has to be recognized, however, that with very small lesions the granuloma, on which histological clasification (as opposed to diagnosis) is mainly based, may be absent, or if present it may be missed in an individual section The chances of finding a granuloma are considerably increased by the cutting of serial sections but this study was based on the examination of small num

bers of sections The chances of success would of course be still further increased by a second biopsy (2) The other possibility is that, although most of the patients claimed to be untreated, some of the lesions may have been histologically indeterminate because they were in regression In this event residual clinical stigmata might be expected to outlast the granulomata

The demonstration of precipitating antimycobacterial antibodies in some of the clinically tuberculoid/borderline patients is in contrast to the findings in the strictly indeterminate group and provides further confirmation that in this group the infections are developed beyond the indeterminate stage Thus, immunological investigation support the value of clinical classification in the early stages of the evolution of leprosy

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INFLUENCE OF SERUM ON THE BACTERICIDAL ACTIVITY OF NEUTROPHIL GRANULOCYTES

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The influence of serum on the intracellular killing of bacteria by neutrophil granulocytes has been examined using a method which facilitates a precise in vitro evaluation of the phagocytic and bactericidal activities of polymorphonuclear leucocytes In the absence of serum a marked reduction in intracellular killing of bacteria was observed Serum markedly enhances the bactericidal activity of neutrophil granulocytes

Phagocytosis and intracellular killing of bacteria are important physiological functions of neutrophil granulocytes For significant phagocytosis to occur, attachment of the bacteria to the phagocytic cell membrane by specific antibodies is required (4 5 6 12) Whether serum factors also stimulate intracellular killing of bacteria by the granulocytes is poorly understood

To study this problem analyses of both phagocytic and bactericidal activities of the granulocytes are required Many of the studies in the past however have been confined to the phagocytic activity and little attention has been paid to the dynamics of the intracellular phase (for review see 1 3 5) A major problem has remained the separation of extracellular and intracellular bacteria in an *in vitro* phagocytic system in order to evaluate the intracellular bactericidal process (2 7 8 9 10 11)

In a previous study (9) it was demonstrated that phenylbutazone effectively prevented killing of phagocytized bacteria by the granulocytes and that intracellular bacteria were protected from the antibacterial effect of penicillin G and streptomycin even in concentrations that killed more than 98 per cent of extracellular organisms in less than 15 minutes Based on these findings a new method for the determination of the number of viable intracellular bacteria has been developed using high concentrations of antibiotics for the inactivation of extracellular bacteria and phenylbutazone for the inhibition of intracellular killing of bacteria (7, 8) This method facilitates a precise *in vitro* evaluation of the phagocytic and bactericidal activities of neutrophil granulocytes (7, 10)

Using this method the influence of serum on the intracellular killing of bacteria by neutrophil granulocytes has been studied

MATERIALS AND METHODS

Leucocytes

per ml saline) by centrifugation at 500 g for 5 minutes (7) After the final centrifugation a differential count was performed and the cells were resuspended in Hanks balanced salt solution containing 0 1 per cent gelatin to make concentra

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tions of 10^7 neutrophils per ml Ninety five to 99 per cent of the isolated neutrophils resisted staining with trypan blue, and their functional integrity was intact as measured by latex particle phagocy tosis Erythrocyte contamination in 50 consecutive specimens varied from 17 to 59 per cent (mean 45 per cent), eosinophil granulocyte contamination from 0 to 9 per cent (mean 4 per cent), basophil granulocyte contamination from 0 to 2 per cent (mean 1 per cent), and lymphocyte monocyte con tamination from 10 to 21 per cent (mean 16 per cent) Platelet contamination was minimal and less than 0 02 per cent of autologous serum remained

Bacteria

Staphylococcus aureus Oxford (Heatley strain obtained from the National Collection of Type Cultures, Colindale London 1958) was used as the test organism (7) The bacteria were cultured overnight in Penassay broth (Difco), twice washed in 0 45 per cent saline and suspended in Hanks balanced salt solution to an optical density of 0 6 at 620 nm in a Beckman spectrophotometer (7) This suspension was diluted in Hanks' balanced salt solution containing 0 1 per cent gelatin to a concentration of $8\ 12 \times 10^7$ colony forming units per ml

The minimum inhibitory concentrations of penicillin G and streptomycin to the *Staphylococcus aureus* strain were 0 03 units and 0 6 μg per ml, re spectively (7)

Serum

One ml volumes of pooled fresh normal serum from six adults were stored at −30° C Immediately before each experiment, 1 ml freshly thawed serum was added to 3 ml Hanks balanced salt solution containing 0 1 per cent gelatin

Leucocyte bacteria Suspension

0 5 ml leucocyte suspension 0 1 ml bacteria suspension and 0 4 ml diluted serum in 0 4 ml Hanks balanced salt solution containing 0 1 per cent gelatin were added to 12×75 mm disposable plastic tubes This provided about 2 bacteria per neutrophil granulocyte and in the tests with se rum a final concentration of 10 per cent serum The tubes were incubated at 37° C with an end over end rotation to promote contact between bac teria and leucocytes Samples were removed at prescribed intervals for determinations of the total number of viable bacteria and the number of viable intracellular bacteria The bactericidal capacity of the granulocytes is proportional to the total num ber of bacteria killed and inversely proportional to the total number of viable bacteria or number of viable intracellular bacteria (7) The number of bacteria phagocytized equals the number of viable intracellular bacteria plus the number of bacteria killed (7)

The total number of viable bacteria was deter mined by adding 0 01 ml of the leucocyte bacteria suspension to 1 ml distilled water to facilitate os motic disruption of the leucocytes Quantitation of viable bacteria was made from appropriate dilu tions of this suspension using a standard pour plate technique and Penassay agar (Difco)

The number of viable intracellular bacteria was determined by the technique of *Solberg* (7) 0 01 ml of the leucocyte bacteria suspension and 1 ml Hanks' balanced salt solution containing 0 1 per cent gelatin, 500 μg streptomycin 500 units peni cillin G and 2 mg phenylbutazone were incubated at 37° C for 15 minutes and centrifuged for 10 minutes at 500 g The cellular pellet was twice washed in 5 ml Hanks' balanced salt solution by centrifugation at 500 g for 10 minutes and re suspended in 1 ml distilled water for osmotic dis ruption of the leucocytes to occur Quantitation of viable bacteria was made by the standard pour plate technique

Controls

Controls consisted of tubes with bacteria and 10 per cent serum without leucocytes to demonstrate any direct bactericidal effect of the serum and tubes with leucocytes and bacteria incubated with out rotation to determine extracellular bactericidal activity for example by enzymes liberated from damaged granulocytes During incubation for 2 hours no reduction in viable bacteria was observed in the control tests

RESULTS

Experiment I

Granulocyte bacteria suspensions with and without serum were incubated at 37° C and the total number of viable bacteria and the number of viable intracellular bacteria were determined at 0, 5, 15, 45 90 and 120 mi nutes

In the test containing serum rapid phagocytosis and killing of bacteria occurred (Fig 1) After 2 hours incubation of the test containing no serum only minor reduction in the total number of viable bacteria was observed, and the number of viable intra cellular bacteria increased slowly during the early phase of incubation and later remained fairly constant, indicating that both phagocytic and bactericidal activities of the granulocytes were markedly reduced

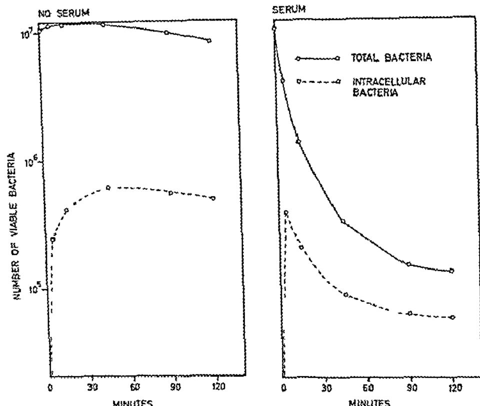


Fig 1 Viable bacterial counts during incubation of granulocyte bacteria suspensions with and without serum (mean of five experiments)

Experiment 2

To substantiate the evidence that lack of serum factors resulted in reduced intracellular killing of bacteria by the leucocytes, the granulocyte-bacteria suspensions were incubated at 37° C for 8 minutes At this time of incubation the numbers of viable intracellular bacteria were about equal in the tests with and without serum (see Fig 1) Cell associated bacteria i e bacteria located intracellularly or attached to the external cell wall were separated from the extracellular organisms and washed three times in Hanks' balanced salt solution by centrifugation at 100 g for 5 minutes, and the cellular pellet was resuspended in 1 ml Hanks' balanced salt solution containing 0 1 per cent gelatin Tubes containing 100 units penicillin G and 100 µg streptomycin and tubes without antibiotics as indicated in Fig 2 were incubated at 37° C Samples (0 01 ml) were removed at prescribed intervals, twice washed in Hanks' balanced salt solution and resuspended in 1 ml distilled water for osmotic disruption of the leucocytes to occur Viable bacteria were counted by the pour-plate technique

In contrast to the test with non-opsonized bacteria and no antibiotics, significant reduction in viable bacteria was observed in the test with opsonized bacteria and no antibiotics demonstrating the increased intracellular killing of bacteria by the granulocytes in the presence of serum factors (Fig 2) In the test with non-opsonized bacteria and antibiotics, significant reduction in viable bacteria was observed during the early phase of incubation due to inactivation by the antibiotics of contaminating extracellular bacteria 7, 9) Later the number of viable bacteria

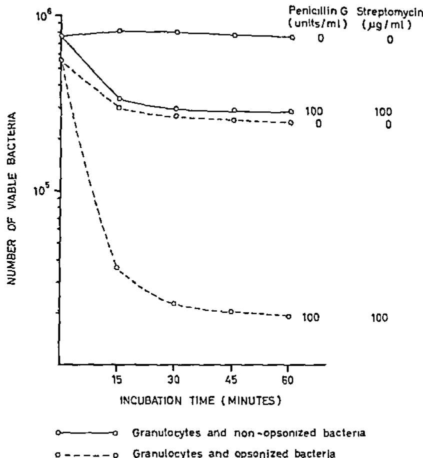


Fig 2 Intracellular killing of opsonized and non opsonized *Staphylococcus aureus* by neutrophil granulocytes (mean of five experiments)

was only slightly reduced, demonstrating poor intracellular killing of bacteria by the granulocytes In the test with opsonized bacteria and antibiotics, a marked reduction in viable bacteria was observed due to the added effects of inactivation of extracellular bacteria by antibiotics and intracellular killing by granulocytes in the presence of serum factors

DISCUSSION

A number of studies have been devoted to the interactions of bacteria and polymorphonuclear leucocytes (1 3 5) However differences in experimental design and difficulties of methodology have resulted in divergent interpretations of experimental results A major problem has remained the elimination of non phagocytized bacteria in an *in vitro* phagocytic system in order to study the intracellular bactericidal processes (2, 8, 11) Previous investigators have usually resorted to differential centrifugation for the separation of extracellular and intracellular bacteria This technique is not entirely quantitative and when the ratio of extracellular to intracellular bacteria is high as in our experiment 1 with significantly reduced phagocytosis in the absence of serum extracellular contamination may markedly obscure the determination of the relatively small numbers of viable intracellular bacteria (2 8 11) In addition, bacteria adhering to the external granulocyte wall may not be eliminated by differential centrifugation and if the leucocyte-bacteria suspension as in the present study contains erythrocytes which is usual when the

granulocytes are prepared from blood samples, immune adherence of bacteria to the erythrocytes may also become a major problem

By the method used in experiment 1, extracellular bacteria are effectively controlled by antibiotics which cause no inactivation of intracellular bacteria (9) Control of extracellular bacteria by antibiotics takes, however, 10-15 minutes During this period, killing of intracellular bacteria by the granulocytes may significantly obscure the results (9) Accordingly, inhibition of the bactericidal activity of the granulocytes is a prerequisite for the determination of the number of viable intracellular bacteria while extracellular killing by antibiotics takes place The essence of our method is, therefore, the combined use of phenylbutazone for the inhibition of intracellular killing of bacteria and antibiotics for the control of extracellular bacteria

In experiment 1, the great majority of bacteria in the test containing no serum remained extracellularly However, some bacteria were phagocytized and killed, demonstrating that intracellular killing of bacteria by granulocytes *can* take place *in* the absence of serum Furthermore, the number of viable intracellular bacteria in this test increased slowly during the early phase of incubation and later remained fairly constant, indicating that the rate of intracellular killing of bacteria in the absence of serum was reduced to about the same extent as the phagocytosis

During the early phase of incubation of granulocytes and opsonized bacteria rapid killing of intracellular bacteria occurs This was clearly demonstrated in experiment 2 where the granulocytes in the test suspensions had been exposed to the bacteria for only 8 minutes and still possessed marked bactericidal activity A significant reduction in viable bacteria was observed during incubation of the test with opsonized bacteria (Fig 2, second curve from the bottom) In contrast no reduction in viable bacteria was observed during incubation of granulocytes containing non-opsonized bacteria, indicating that the intracellular killing of bacteria in the absence of serum factors was poor However, the results of these two tests were obscured by contamination with extracellular bacteria This was clearly demonstrated when antibiotics were added to the test suspensions to eliminate extracellular bacteria In the test with granulocytes containing non-opsonized bacteria, significant reduction in viable bacteria was observed during the early phase of incubation due to killing of extracellular bacteria by the antibiotics (9) Later, however, the reduction in viable bacteria was insignificant compared to the marked reduction in the test with granulocytes containing opsonized bacteria Accordingly, serum factors stimulate not only the phagocytosis of bacteria by the granulocytes markedly but also the intracellular killing of bacteria

The reason for the reduced bactericidal activity for the granulocytes in the absence of serum is unknown However, for significant phagocytosis to occur, attachment of opsonized bacteria to the phagocytic cell membrane by the Fc part of specific antibody molecules is required (4, 5, 6, 12) Once attachment occurs, the leucocyte membrane is sending out microprojections which surround the bacterium and bactericidal enzymes are released into this phagocytic vacuole Probably, the attachment of bacteria to the membrane of the phagocytic vacuole acts as a stimulus for the release of these enzymes This would explain the markedly reduced bactericidal activity of the granulocytes when no attachment occurs in the absence of serum

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ANTIBODIES AGAINST THREE DIFFERENT STRUCTURAL COMPONENTS OF MEASLES VIRUS IN PATIENTS WITH MULTIPLE SCLEROSIS, THEIR SIBLINGS AND MATCHED CONTROLS

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Patients with multiple sclerosis, their siblings and controls matched for age, sex and place of residence were tested for antibodies to measles virus hemagglutinin (HI), hemolysin (HLI) and nucleocapsid complement fixing (NC CF) antigen No qualitative differences between the study groups as concerns the diversity of the antibody responses to measles were found The mean titres of MS specimens were about one logarithm of two higher than the means of the controls in each of the tests The difference was somewhat more pronounced in the HLI and NC CF tests than in the HI test Females had higher titres than males in all of the tests Antibody titres were higher in subjects who had contracted their measles at an older age However this finding could not explain the observed differences in antibody titres since MS patients had higher titres than the controls in all age groups The importance of appropriate selection of measles antibody test for serological characterization of a clinical material of the present kind is emphasized

Results of numerous studies indicate that patients with multiple sclerosis (MS) have significantly higher measles antibody titres in their serum specimens than the normal population or matched controls, whereas no corresponding differences can be demonstrated in tests for antibodies against several other viruses (5, 11) Various tests for measles antibodies have been used, but in most cases their structural correlates in measles virus have not been defined Recently tests have been developed for the identification of antibodies to different structural components of measles virus (10) The antibody responses to measles virus hemagglutinin, hemolysin and nucleocapsid components have been found to vary quantitatively and qualitatively in acute and chronic measles infections (9, 13)

The purpose of the present study was to test specimens from MS patients, their siblings, and matched controls for antibodies to the above mentioned three different structural components of measles virus The same clinical material was previously used for

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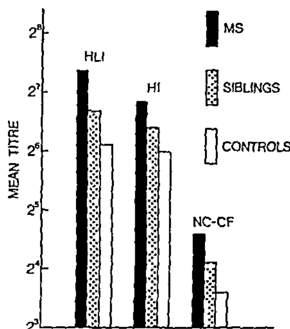


Fig 1 Schematic presentation of the mean antibody titres in different tests in the MS, sibling and control groups

studies by certain tests for antibodies against different viruses, including measles (11) The differences between the study groups were analysed and the titres of the measles virus antibodies of the entire material and in the separate study groups were also correlated Previous experience of ordinary measles and the clinical data of the MS patients were correlated with the results of the antibody tests

MATERIAL AND METHODS

Serum specimens The serum material has been described in detail in an earlier report (11) For the present study 180 MS control pairs were available and 115 specimens from siblings of the patients were also included in the test series The controls were matched for the age by one year, sex and place of residence The age of the sibling was within 5 years of that of the patient For technical reasons all serum specimens were not included in all of the different tests The tests were done under a code number in one laboratory and the results analysed in the other The subjects were interviewed for the time at which they had contracted measles and this information was checked with other family members by a trained nurse

Serological techniques Three techniques were employed to measure antibodies to measles virus These have been described in detail earlier (9)

The hemagglutination inhibition (HI) tests were performed by use of a micro technique with four hemagglutinating units of Tween 80 ether treated antigen prepared in primary dog kidney cells (8)

The hemolysis inhibition (HLI) test has been described in detail earlier (9) The antigen was concentrated supernatant from measles infected VERO cells The hemolysing effect of this antigen on red blood cells from green monkey was inhibited by measles antibodies The highest dilution of the serum giving at least 50 per cent inhibition of the hemolysing activity as measured at 540 nm in a spectrophotometer was the titer of the specimen It should be mentioned that under the conditions of testing HI antibodies gives HLI, whereas antibodies reacting with the hemolysin do not inhibit the hemagglutinating activity of Tween 80-ether treated antigen (9)

The complement fixation test for antibodies to nucleocapsids (NC CF) was performed with a micro technique The antigen was a partially purified nucleocapsid material of measles virus (10) Four antigenic units and two full units of guinea pig complement were employed in the tests

Analysis of the results The results were punched on cards and analysed in a Univac 1108 computer by statistical package programmes developed in the Computer Center, University of Helsinki The means of the titres were calculated with the natural logarithms of the titre reciprocals and the means were expressed as logarithms of 2 The significance of the differences found between the means was tested in the Student's t test The titre values in each pair (MS control, MS-sibling and sibling control) were also compared to each other and the significance of the deviations from binomic distribution was tested after z transformation in the sign test Analysis of variance was also performed with some of the results

RESULTS

Occurrence of antibodies to different measles virus components in MS patients their siblings and matched controls The geometric means of antibody titres in the entire material measured by measles HI, HLI and NC-CF tests are shown in Fig 1 In all of the tests significantly higher ($p < 0.001$) mean titres were found in the MS group as compared with the group of controls However the differences are only about one logarithm of 2 The mean values of siblings were between those of MS patients and controls Separate

analysis of the mean antibody titres in males and females revealed similar results, although females in all the groups had higher mean titres than the males (Table 1) The mean titres of females of the sibling group were slightly higher in all the tests than those of the males in the MS group The material was also analysed after separation into groups from high, medium and low MS risk areas of Finland (11) Differences corresponding to those found in the entire material were found between MS and control groups in each of the risk areas

Since a few extremely high titres may influence the mean titres and make them statistically unreliable (5), the titres in the MS group were compared with the corresponding titres in the matched control groups The results were expressed as percentage of higher, equal or lower titres in MS patients as compared with the controls (Table 2)

TABLE 1 *Measles Antibody Titres in Multiple Sclerosis Patients, Their Siblings and Matched Controls*

Group	Males Geometric mean* antibody titres in			Females Geometric mean* antibody titres in		
	HI	HLI	NC CF	HI	HLI	NC CF
MS	6 5§	7 0†	4 5†	7 2†	7 9†	5 2†
Siblings	6 1	6 2	3 9	6 8	7 4	4 6
Controls	6 0	5 8	3 3	6 1	6 4	4 2

TABLE 2 *Number of MS Control Pairs with Higher, Lower or Equal Antibody Titres*

Measles antibody test	MS higher	Control higher	Equal
HI	99*	49*	27
HLI	106*	36*	27
NC CF	105*	30*	30

* Highly significant differences (p < 0 001) in sign test.

TABLE 3 *Number of MS Sibling Pairs with Higher, Lower or Equal Titres*

Measles antibody test	MS higher	Sibling higher	Equal
HI	49	40	23
HLI	61*	27*	20
NC CF	59§	32§	16

* The difference is highly significant (p < 0 001) in sign test

§ The difference is significant (p < 0 01) in sign test

TABLE 4 *Number of Sibling Control Pairs with Higher, Lower or Equal Titres*

Measles antibody test	Siblings higher	Control higher	Equal
HI	54	37	19
HLI	55*	31*	17
NC CF	54*	33*	16

* The difference is almost significant (p < 0 05) in sign test

When these differences were tested in the sign test, the entire group of MS patients as a whole or separated into males and females had higher titres more frequently than the controls (p < 0 001) Also by this analysis siblings of the patients had a position between the MS patients and the controls in each of the tests (Table 3 and 4)

Correlation between antibody titres measured with different techniques Both the HI and HLI antibody titres and the HLI and NC CF titres were correlated (Tables 5 and 6) About ten per cent of the specimens had an 8 fold or higher excess of HLI over HI antibodies The specimens with HI or HLI antibodies but without NC CF antibodies amounted to about five per cent of the entire material Two or three per cent on the specimens had NC CF antibodies without measurable titres in the HI or HLI tests These observations were valid for the entire material and for separated groups of males and females No special features regarding the relationship between titres of different

TABLE 5 *The Correlation between Measles Hemagglutination Inhibition (HI) and Hemolysin Inhibition (HLI) Titres in the Total Study Material (the Number on Specimens with Indicated Titres are shown)*

Reciprocal HI titre	Reciprocal HLI titre <10	10	20	40	80	160	320	640	1280	2560
<10	5		4	1	1	2	2			
10	2	2	6	6	7	1	3			
20	7	8	7	13	14	4	4	1	1	
40		2	19	20	19	10	4	7	1	
80			10	11	25	23	13	4	1	
160			1	7	21	20	14	8	1	
320					13	23	16	12	4	1
640					1	9	10	8	1	
1280							7	3	2	1
2560								3	1	1

TABLE 6 *The Correlation between Nucleocapsid Complement Fixation (NC CF) and Hemolysin Inhibition (HLI) Titres in the Total Study Material (the Number of Specimens with Indicated Titres are shown)*

Reciprocal NC CF titre	Reciprocal HLI titre <10	10	20	40	80	160	320	640	1280	2560
<5	1	2	5	4	3	1	2			
5	2	5	14	9	11	5		2		
10	3	1	8	8	16	16	4	4	1	
20		1	7	11	23	13	4	4	1	
40		1	3	7	13	15	10	1	3	
80			3	3	4	9	11	8	2	
160			1		3	6	7	6	1	1
320						1		3		1
640										
1280	2			2	2					

TABLE 7 *Regression Lines between Results in HLI Tests and HI or NC CF Tests in the Study Groups*

Group of specimens	Regression lines *HLI/HI*	HLI/NC CF
MS patients	$\log_e(\mathrm{HLI}) = 2\ 8 + 0\ 48 \times \log_e(\mathrm{HI})$	$\log_e(\mathrm{HLI}) = 1\ 9 + 0\ 25\ \log_e(\mathrm{NC\ CF})$
Siblings	$= 2\ 1 + 0\ 57$	$= 1\ 5 + 0\ 30$
Controls	$= 1\ 8 + 0\ 58$	$= 1\ 1 + 0\ 33$

measles antibodies could be found between the MS, sibling or control groups as illustrated by the regression lines presented in Table 7

The effect on measles antibody titres of age of subjects at time of sampling age for contraction of measles and clinical history of MS Because antibody titres generally decrease with increasing age, the MS, sibling and control groups were matched also with regard

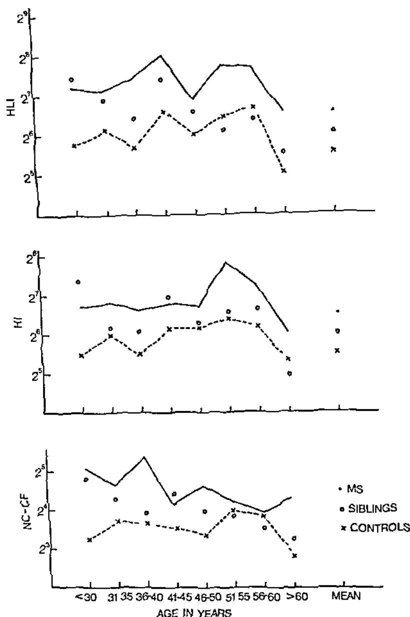


Fig 2 Mean antibody titres in different tests in the MS sibling and control groups subdivided according to age of subjects at time of sampling

to age (*11*) The material was divided into groups by 5 year intervals and geometric means in each of the groups were calculated (Fig 2) The figure demonstrates that MS patients have higher mean titres than the control group in each of the tests and in all of the groups between 30 and 60 years of age Similar differences were found after separation of the material into male and female groups The means of the siblings in most age groups were between the means of the MS patients and the controls There was a certain tendency for siblings of lower age groups to have a mean antibody titre corresponding to

TABLE 5 *The Correlation between Measles Hemagglutination Inhibition (HI) and Hemolysin Inhibi tion (HLI) Titres in the Total Study Material (the Number on Specimens with Indicated Titres are shown)*

Reciprocal *HI* titre	Reciprocal HLI titre									
	<10	10	20	40	80	160	320	640	1280	2560
<10	5		4	1	1	2	2			
10	2	2	6	6	7	1	3			
20	7	8	7	13	14	4	4	1	1	
40		2	19	20	19	10	4	7	1	
80			10	11	25	23	13	4	1	
160			1	7	21	20	14	8	1	
320					13	23	16	12	4	1
640					1	9	10	8	1	
1280							7	3	2	1
2560								3	1	1

TABLE 6 *The Correlation between Nucleocapsid Complement Fixation (NC CF) and Hemolysin Inhibition (HLI) Titres in the Total Study Material (the Number of Specimens with Indicated Titres are shown)*

Reciprocal *NC CF* titre	Reciprocal HLI titre									
	<10	10	20	40	80	160	320	640	1280	2560
<5	1	2	5	4	3	1	2			
5	2	5	14	9	11	5		2		
10	3	1	8	8	16	16	4	4	1	
20		1	7	11	23	13	4	4	1	
40		1	3	7	13	15	10	1	3	
80			3	3	4	9	11	8	2	
160			1		3	6	7	6	1	1
320						1		3		1
640										
1280	2			2	2					

TABLE 7 *Regression Lines between Results in HLI Tests and HI or NC CF Tests in the Study Groups*

Group of specimens	Regression lines HLI/HI	Regression lines HLI/NC-CF
MS patients	$\log_e$(HLI) ~ 2 8 + 0 18 × $\log_e$(HI)	$\log_e$(HLI) ~ 1 9 + 0 25 $\log_e$(NC-CF)
Siblings	2 1 + 0 57	1 5 + 0 30
Controls	1 8 + 0 58	1 1 + 0 33

measles antibodies could be found between the MS sibling or control groups as illustrated by the regression lines presented in Table 7

The effect on measles antibody titres of age of subjects at time of sampling age for contraction of measles and clinical history of MS Because antibody titres generally decrease with increasing age the MS sibling and control groups were matched also with regard

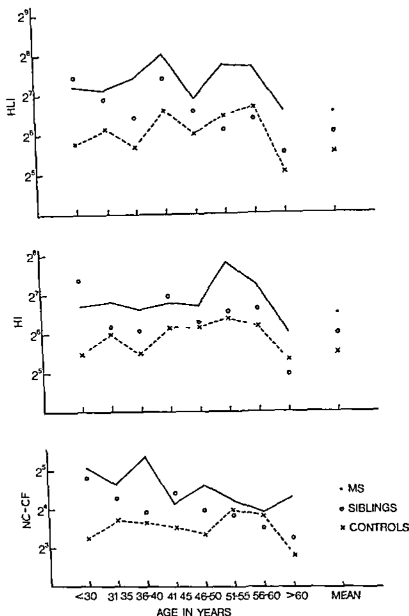


Fig 2 Mean antibody titres in different tests in the MS, sibling and control groups subdivided according to age of subjects at time of sampling

to age (11) The material was divided into groups by 5-year intervals and geometric means in each of the groups were calculated (Fig 2) The figure demonstrates that MS patients have higher mean titres than the control group in each of the tests and in all of the groups between 30 and 60 years of age Similar differences were found after separation of the material into male and female groups The means of the siblings in most age groups were between the means of the MS patients and the controls There was a certain tendency for siblings of lower age groups to have a mean antibody titre corresponding to

TABLE 8 *Age at which Measles was Contracted and Antibody Levels*

Age at which measles was contracted (years)	HI Mean§	HI SD†	HI No	HLI* Mean§	HLI* SD†	HLI* No	NC CF Mean§	NC CF SD†	NC CF No
Under 5	6 3	2 1	120	6 4	2 1	118	4 4	1 9	115
6 10	6 6	1 7	166	6 9	1 7	156	4 3	2 0	159
11 15	6 8	2 0	28	7 4	1 7	28	4 3	1 8	28
16 or older	7 6	2 8	14	7 9	2 2	14	5 1	2 0	14

* Statistically almost significant difference between age groups in analysis of variance ($p < 0\ 05$)
§ Means expressed as logarithms of 2
† S D means standard deviation

that of MS patients and of higher age groups to have mean titres more similar to those of the controls However the small size of the different age groups preclude an analysis of the significance of this trend

The subjects in this study were interviewed for their past measles history Acceptable information was obtained from about 75 per cent of the study population (11) The material was divided into groups (5 year intervals) according to the age at which the measles was contracted In Table 8 are shown the calculated mean titres in the three measles antibody tests In all the tests higher means were seen in subjects who contracted measles at higher age than in those who had the disease during childhood This tendency was apparent both for MS and control groups when tested separately

The MS patients were divided into groups according to the year of the beginning of the disease to the course of the disease and to the disability grade resulting from the disease No differences were found between these groups in any of the measles antibody tests

DISCUSSION

This report gives supplementary information about measles antibody levels in an earlier described collection of serum specimens from MS patients their siblings and matched controls (11) The MS patients had significantly higher titres [illegible] n the controls in three tests for antibo[illegible] against separate structural components of measles This difference was not age dependent but was found in all age groups between 30 and 60 years The statistical analyses were carried out by use of the parametric t test and the nonparametric sign test to show statistical differences Although the latter test is rather ineffective in distinguishing between groups of results since it takes into consideration only the direction of the differences highly significant differences were found in each of the tests between MS and control groups The siblings had higher titres than the controls and females in each of the study groups higher titres than the males

It has been suggested that the differences in measles antibody levels between MS patients and controls may be due to a common antigenic determinant in measles virus and in the encephalitogenic factor of myelin (6) This study however, demonstrated that antibodies to three separate structural components of measles are elevated in MS patients To explain these findings one has to assume that measles virus antigen is involved in the process which leads to the increase of antibody levels We have earlier shown that active antibody production against measles virus occurs in the central nervous system (CNS) of a certain fraction of MS patients and of all SSPE patients (13) The slight differences found in serum measles antibody levels (about one logarithm of two) between MS and control patients possibly could be explained by a contribution of CNS derived

antibodies to the serum levels This hypothesis implies that there is or has been an immunization by measles antigen in the CNS of some MS patients This antigen stimulus could be represented (a) by virus antigen which is passively released during the demyelinization process (b) by a pronounced and long lasting immunization in connection with the ordinary acute measles virus infection or (c) by antigen produced through activation of a latent measles infection More detailed virological studies on various materials from MS patients are needed to allow a choice between these possibilities

The intermediate position of the mean antibody titres of siblings in each of the tests is difficult to explain Also earlier studies have suggested that siblings have higher measles titres than controls (1 4) It is possible that some genetic factors may play a role in this phenomenon since it has been demonstrated that MS patients have a different HL-A antigen pattern than the controls (2) The susceptibility to certain virus infections also is linked with the HL-A antigen pattern (3) These genetic differences could lead to a slightly different course of the measles infections and possibly result in higher antibody levels in some families The factors responsible for the establishment of a latent measles infection as well as for the activation of this may also be linked in same way to some genetic factors The possibility is not excluded that slight variations of wild measles virus strains could cause differences in antibody responses and in frequency of persistent infections although by available immunological criteria only one measles virus variant has been demonstrated

The observation that females in all the measles antibody tests have higher mean titres than the males have previously been made by others (1 7) but the basis for this phenomenon is not known Similar observations have also been made in studies of antibody responses to Epstein Barr virus and to certain bacterial vaccines (*J Nikoskelainen* personal communication 12) Thus it is important that the control groups are selected according to sex It is further of importance that controls show an age distribution corresponding to that of MS patients since subjects who contracted measles later in life displayed relatively higher measles antibody titres It should be mentioned that althougn there was a slight tendency for MS patients to contract their measles infection later in life than controls (11) this could not explain the differences observed between antibody titres

A somewhat more pronounced diversity of the antibody response to different measles virus components in samples from SSPE patients and from some MS patients than from cases of ordinary measles was found in earlier studies (9, 13) In this study no qualitative differences between the antibody response in MS and control groups could be found Thus for example the relative occurrence of subjects with significantly higher HLI than HI antibody titres was similar in the MS sibling and control groups (Table 7)

The present results emphasize the importance of using several tests which measure antibodies to different antigenic components of viruses in studies of this kind The HI test is the one most commonly used to measure measles antibodies but the difference in means between the various groups were more pronounced in both the HLI and NC CF tests than in the HI test Concerning the usefulness of HLI tests vs HI tests the following comments can be made Antibodies demonstrated in the HLI test include those reacting with the hemagglutinin as well as with one or more other envelope components For this reason the titre of HLI (or neutralizing) antibodies represent a better indicator of the overall occurrence of antibodies against the envelope than HI antibodies

The quality of the type of CF antigen employed appears to be important No differences between the MS and control groups of the present material (11) could be shown in an earlier study with a crude measles CF antigen However, a significant difference in mean antibody titres of the CF test between the groups was found in this study by use of

a partially purified nucleocapsid CF antigen It has been shown earlier that nucleocapsids represent one of the main CF antigens in measles virus (9), but that envelope components also can be identified by complement fixation (10) The relative amounts of these antigens and possible nonstructural CF antigens of measles may vary in different batches of crude CF antigen preparations

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RABBIT ANTI RAT LYMPHOCYTE SERUM: IMMUNOSUPPRESSION MEDIATED BY A PURE IgM FRACTION

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Eleven rabbits were immunized i v weekly for 3 weeks with rat thymus cells during azathioprine administration The antisera were pooled and fractionated on Sephadex G 200 to obtain IgM and IgG fractions The lymphocytotoxic activity was stronger in the IgM than in the IgG fraction Whole serum, the IgM fraction and the IgG fraction prolonged the survival time of skin allografts from 12 0 to 16 5, 19 2 and 14 4 days respectively It is concluded that IgM antibodies in antilymphocyte sera also can exhibit immunosuppressive activity

Crude antisera raised in rabbits treated with azathioprine during the course of immunization with rat lymphocytes, have been shown to be immunosuppressive (4) These antisera contained most of the lymphocytotoxic activity in the IgM fraction as judged by in vitro tests Both lymphocytotoxic and immunosuppressive activity were lost by reduction and alkylation, suggesting that IgM antibodies were responsible for the immunosuppressive effect

In the present study, these experiments have been repeated and extended by fractionation of crude sera The immunosuppressive effect of pure IgM and IgG fractions were studied and the results showed that IgM antibodies of such antisera have immunosuppressive properties

MATERIALS AND METHODS

Preparation of antisera Thymus cells from hooded BDF rats were prepared as described previously (4) Eleven rabbits were immunized intravenously with 0 9-1 2 $\times$ 10^8 viable cells three times at weekly intervals, i e on days 1,8 and 15 The rabbits were given daily subcutaneous injections of azathioprine, 6 mg/kg body weight, into the loose tissues of the neck from day 1 to day 17

The rabbits were bled approximately 50 ml from the ear vein under Nembutal anaesthesia on day 19, and then by heart puncture on day 20 Aliquots from each bleeding were kept separate for in vitro studies The remaining sera were then pooled and inactivated by heating to 56° C for 30 min Absorption of 600 ml antiserum with 20 ml packed rat red blood cells, washed three times in 0 15 M NaCl, brought the haemagglutination titre to 4 The pooled batch was then stored at −20° C

In Vitro Studies

Gel filtration chromatography was carried out using a 125 cm $\times$ 6 15 cm Sephadex G-200 column Approximately 50 ml pooled serum was applied to the column at each run. Flow rate was adjusted to 48 ml/hour and fractions collected every 20 min Protein concentration in the effluent was monitored in a Uvicord photometer at 254 nm (LKB, Stockholm, Sweden) In the first run, a trace amount of ^{125} Iodine labelled human IgM was added to the rabbit serum before appl on the column to control the efficiency of the paration procedure The radioactivity of the

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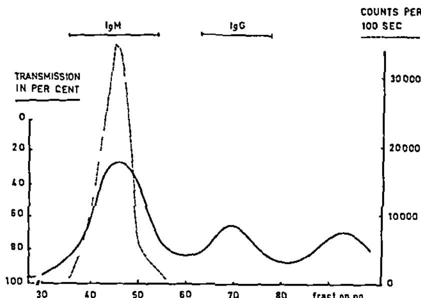


Fig 1 Separation of whole rabbit anti rat lymphocyte serum on Sephadex G 200 The extinction curve (——) from a monitor has been compared with the radioactivity curve (– – – –) following application of a sample containing a trace amount of ^{125} iodine labelled human IgM The horizontal bars at the top indicate pooled fractions

tions was determined and compared to the extinction curve indicating the protein concentration of the effluent The radioactive protein was confined to the first peak as shown in Fig 1, indicating a satisfactory separation From each run, the individual fractions of the first peak designated the IgM fraction and of the second peak, designated the IgG fraction were pooled and then concentrated by pressure dialysis A total of 350 ml antiserum were chromatographed in 8 runs

Agar diffusion tests The following antisera were used [1])Goat anti rabbit IgM (Nordic Pharmaceuticals and Diagnostics Tilbug the Netherlands batch no 570) In immunoelectrophoresis the antiserum gave a single line against whole rabbit serum with form and position characteristic of IgM It did not cross react with isolated rabbit IgG nor with pepsin split rabit IgG [2])Sheep anti rabbit IgG (Dakopats Copenhagen Denmark lot No 029) The antiserum precipitated rabbit IgG but not pepsin split rabbit IgG and did not cross react with rabbit IgM in double diffusion tests in agar

In double diffusion tests in agar all IgM pools precipitated strongly with anti IgM, but did not precipitate with anti IgG The IgG pools precipitated strongly with anti IgG none of them precipitated with anti IgM The IgM pools of the 8 different runs were then pooled to a final pool and concentrated and the IgG fractions were treated similarly This IgM pool again precipitated with anti IgM but no reaction with anti IgG was seen in double diffusion tests in agar

The IgM concentrations of the original anti lymphocyte serum pool and the final concentrated IgM preparation were determined by single radial diffusion using the same anti IgM antiserum the relative amounts of IgM of whole serum and of IgM fraction were as 100 to 130 The original serum volume was 350 ml which on fractionation and concentration gave 260 ml IgM solution and 100 ml IgG solution This indicated little if any loss during the gel filtration procedure the ratio between the volumes (260 350 = 100 134) being similar to the relative concentration of IgM 0 75 ml IgM solution was therefore considered to correspond to 1ml whole serum as regards content of IgM antibodies A technical failure made the quantitation of IgG unreliable To use equivalent amounts of IgG antibodies during in vivo experiments it was assumed that the recovery of IgG was equal to that of IgM during gel filtration 0 3 ml IgG solution was thus taken to correspond to 1 ml whole serum as regards content of IgG antibodies (100ml 350ml = 0 29)

The IgM and IgG pools were dialysed three times against 50 volumes of phosphate buffered saline for 24 hours to remove active metabolites of azathioprine

The lymphocytotoxic test This was carried out as described previously using Trypan Blue (E.T Gurr London England) guinea pig serum as source of complement and peripheral blood lymphocytes as target cells (2) Crude serum ME treated serum the IgM and the IgG pools were

TABLE 1 *In Vitro Lymphocytotoxic Activity of Crude Rabbit Anti Rat Lymphocyte Serum and Its IgM and IgG Fractions before and after Reduction and Alkylation*

	Crude serum	IgM fraction	IgG fraction
Untreated	32	16	4
After reduction and alkylation	<4	<4	<4

tes es in twofold serial dilutions According to the findings above 0 75 ml IgM pool diluted to 1 ml with Hanks solution and 0 3 ml IgG pool diluted to 1 ml with Hanks solution were used as reference when the immunoglobulin fractions were compared with crude serum

Mercapto ethanol (ME) treatment This was carried out using a final concentration of 0 1 M 2—mercapto—ethanol for 2 hours at room temperature followed by alkylation with 0 02 M iodoacetamide in 0 006 M phosphate buffer pH 7 2 as described previously (2)

In Vivo Studies

Administration of antilymphocyte serum and fractions thereof Crude serum pure IgM and the IgG fractions were given according to the following schedule

2 ml crude serum 1 5 ml IgM fraction or 0 6 ml IgG fraction from day −7 to day 1 1 ml crude serum 0 75 ml IgM fraction or 0 3 ml IgG fraction daily from day 0 (the day of grafting) to day +7 Combined treatment consisted of 1 5 ml IgM + 0 6 ml IgG initially followed by 0 75 ml IgM + 0 3 ml IgG according to the same schedule

Skin grafting technique Full thickness skin grafts were transplanted from inbred Fischer male rats to inbred hooded BDE male rats as described previously (3) Six groups of rats were used untreated controls (10 animals) were compared with rats obtaining crude ALS (2 animals), ME treated crude ALS (2 animals), the IgM fraction (5 animals) the IgG fraction (5 animals), and the IgM plus the IgG fraction (3 animals) respectively

RESULTS

The pooled antiserum had a low lymphocytotoxic titre (Table 1), but the greater part of this activity was located in the IgM fraction The IgG fraction had a titre of 4, the IgM fraction a titre of 16 Both the crude serum, the IgG, and the IgM fraction lost in vitro lymphocytotoxic activity by treatment with 0 1 M 2 mercapto- ethanol followed by alkylation with iodoacetamide

Skin Graft Survival

Data from the skin grafting experiments are shown in Table 2 and Fig 2 The crude pooled antiserum prolonged the survival time from 12 0 ± 0 45 days (mean ± S D) to

TABLE 2 *Survival Times of Skin Allografts in Rats (Fischer to Hooded BDE) Treated with whole Rabbit Anti Rat Lymphocyte Serum and Fractions* thereof, Compared to Treatment with Normal Rabbit *Serum and no Treatment*

Type of treatment	No of animals	Survival times: Range	Survival times: Mean ± S D	Student T test
No (control)	10	11 13	12 0 ± 0 45	
Normal rabbit serum	5	10	10 0	p < 0 01 versus control
Whole serum	2	16–17	16 5 ± 0 5	p < 0 001 versus control
Reduced and alkylated whole serum	2	11	11 0	p > 0 1 versus control
IgM fraction	5	16 22	19 2 + 2 58	p < 0 001 versus control p 0 05 versus whole serum
IgG fraction	5	13–15	14 4 ± 0 89	p < 0 001 versus control p < 0 0025 versus IgM p = 0 025 versus whole serum
IgM + IgG fraction	3	19 21	19 5 ± 1 29	p < 0 001 versus control p < 0 001 versus IgG p > 0 1 versus IgM

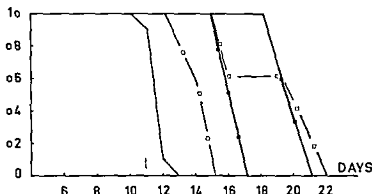


Fig 2 Survival curves for skin allografts from Fischer to hooded BDE rats Comparison of untreated controls with rats given whole serum reduced and alkylated serum or fractions thereof —— untreated —●—● whole serum reduced and alkylated serum —□—□— IgM fract on —○— ○— IgG fraction —■—■— IgM + IgG fraction

16 5 ± 0 5 days This difference was statistically significant ($p < 0\ 001$) Mercapto ethanol treatment and subsequent alkylation of the crude serum resulted in loss of immunosuppressive activity (survival time 11 0 ± 0 5 days ($p < 0\ 1$ versus control)) Treatment with the pure IgM fraction increased the survival time of skin allografts to 19 2 ± 2 58 days which is a statistically significant difference from the control value $p < 0\ 001$ The survival time was slightly longer than that obtained with the crude serum 19 2 versus 16 5 days but this difference is not statistically significant ($p - 0\ 05$) Treatment with the IgG fraction prolonged graft survival to 14 4 ± 0 89 days which again is significantly different from untreated controls ($p < 0\ 001$) but not from treatment with crude antilymphocyte serum ($p - 0\ 025$) Treatment with the IgM fraction prolonged the survival time more than treatment with the IgG fraction (19 2 ± 2 58 versus 14 4 ± 0 89 days) this difference is statistically significant ($p < 0\ 0025$) The combined use of IgM and IgG gave a similar prolongation as with IgM alone (19 5 ± 1 29 days) This is significant versus control and IgG but not versus crude serum or IgM alone

DISCUSSION

In a previous investigation (4) it was found that crude antilymphocyte sera raised in rabbits treated with azathioprine during the immunization course lost their in vitro and in vivo activity after treatment with 2 mercapto-ethanol followed by alkylation This suggested that the immunosuppressive activity was mediated by IgM antibodies

The present study with a similar but not identical antiserum pool confirmed this Isolated IgM antibodies of rabbit ant rat lymphocyte sera (ALS) raised during azathioprine treatment were able to prolong the survival time of skin allografts Isolated IgG antibodies from the same antisera were less efficient than IgM antibodies A mixture of IgM and IgG antibodies had immunosuppressive activities similar to those of pure IgM

These findings are in contrast to the general view locating all immunosuppressive activity to the IgG fraction (5 6 7 *Mandel* & *Asofsky* have previously demonstrated some but definite immunosuppressive activity of the IgM fraction (8) The bulk of the immunosuppressive activity of their ALS was however located in the IgG fraction Since

TABLE 3 *Comparison of Two Pools of Rabbit Anti Rat Lymphocyte Sera Raised during Azathioprine Treatment*

Cell dose	Pool I* 0 8 – 1 4 × 10⁹	Pool II 0 9 – 1 2 × 10⁹
Duration of azathioprine treatment	22 days	17 days
Day of bleeding after last immunizing dose	10 days	4 and 5 days
Lymphocytotoxic titre of whole serum	256	32
Lymphocytotoxic titre after reduction and alkylation	16	<4
Graft survival in recipients treated with whole serum (mean and range)	19 0 (17–21)	16 5 (16–17)
Graft survival in untreated recipients	12 0 (11–13)	12 0 (12–)

* Pool I refers to antisera studied previously (4) Pool II refers to sera studied in the present report.

Mandel & Asofsky used a graft versus host system to assay immunosuppressive activity, a system which is probably easier to suppress than skin allografts, the present results illustrate unequivocally that IgM antibodies may have strong immunosuppressive activity

The present antiserum pool differed somewhat from that of the previous study (Table 3) A similar cell dose and immunization schedule was used, but the rabbits were bled on day + 4 and 5 compared to day + 10 after the last immunization The azathioprine treatment was accordingly of shorter duration (17 versus 22 days) The period before bleeding the rabbits was shortened to obtain antisera with maximal content of IgM and with as little activity in the IgG fraction as possible Certain in vitro characteristics suggest that this was achieved Though treatment with mercapto-ethanol reduced the lymphocytotoxic titre of both pools of antisera by 4 double dilutions only the serum used in the present report lost all activity

These two pools prolonged the survival time of Fischer skin grafts to hooded BDE rats The difference between the graft survival times (19 0 versus 16 5 days) is not statistically significant This may partly be due to only two animals being used to assay the in vivo properties of the latter pool while ten animals were used for the previous pool If however the difference in survival times was real this would allow some speculation The two serum pools only differ in the time of bleeding the serum raising animals Pool I, with the longest period before bleeding had a higher in vitro titre after reduction and alkylation this suggests more IgG activity The difference in graft survival times could thus result from increased IgG in vivo active antibodies, either acting separately or in synergism with IgM antibodies *Mandel & Asofsky* (8) demonstrated synergistic activity between IgM and IgG antibodies of their ALS *Bradley & Barnes* (1) found that a combination of certain early and late antisera resulted in increased survival times

By azathioprine treatment of the serum raising animal, immunosuppressive activity was located in another immunoglobulin fraction than usual The rigid concept that immunosuppressive activity is only located in the IgG fraction of antilymphocyte sera is thus no more tenable

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IMMUNODIFFUSION STUDIES ON SOME *PROTEUS* STRAINS

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By means of double diffusion in gel, immunoelectrophoresis and crossed immunoelectro phoresis some *Proteus* strains were examined In the strain O3 H1 the O3, H1 and a protein antigen common to many members of *Enterbacteriaceae* were identified An addi tional 30 40 antigens were noted No K antigen could be discerned Forty nine *Proteus* strains of eleven different O groups isolated from patients showed a very similar electro-phoretic mobility of the O antigens as compared to the O antigens of *Proteus* standard strains.

On the basis of the classical serology of the *Enterobacteriaceae* as described by *Kauffmann* (18), many studies concerning the antigens of *Salmonella* and *E coli* have been performed by means of immunodiffusion techniques (9, 11, 12, 19) Investigations of *Proteus* antigens using these methods are scarce (3, 14) and few epidemiological studies of *Proteus* O serotypes have been performed (20, 21, 24)

The purpose of the present study was to obtain a picture of the antigenic pattern of *Proteus*, to serve as a basis for studies of the host parasite relationship in urinary tract in fections, UTI, caused by *Proteus* bacteria In this work, *Proteus* includes *P mirabilis* and *P vulgaris* using the serological system of *Kauffmann & Perch* (18) *P morganii* and *P rettgeri* are not included in this sy-stem as they are referred to as separate gene-ra by *Kauffmann* (18) Also they are not so frequently isolated from man

MATERIALS AND METHODS

Bacterial Strains

The following standard strains described by *Perch* (29) and kindly supplied by Dr *H Gnarpe*, Uppsala, Sweden, were used (Table 1)

All of the strains showed swarming phenomena on Drigalski agar plates made as described by *Kauffmann* (18) with the exception that nutrient agar was used instead of placenta broth and Danish agar No strain gave autoagglutination when boiled for 2½ hours

Proteus strains were isolated from patients with significant bacteriuria ($\geq$100,000 bacteria/ml of urine) from septic patients with positive blood cultures and from the stools of healthy individuals O grouping was performed as described by *Lincoln* (23) Only strains belonging to the O groups men-tioned above were included

For the more detailed antigenic analyses the strain O3 H1 was chosen as model strain since this O antigen is very often found in strains from pa tients with infected wounds or urinary tract infec-tions (21, 32)

Crude Antigen Preparations Used for Immunodiffusion Analyses

The strains were inoculated on CLED agar plates (Oxoid) overnight Single bright colonies were selected as inocula and were cultivated on 0 75 per cent nutrient agar plates for 18 24 hours at

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Table 1.

Designation	Subgenus	Serotype			
		Extended		Simplified	
		O	H		
XL	1*	1a, 1b	1a 1b, 1d	O1 H1	
X2 3307 N C	1	2a	1a, 1b, 1c	O2 H1	
XK	2	3a, 3b	1a, 1c, 1e	O3 H1	
F 403	2	3a, 3b	2a, 2b, 2e, 2f	O3 H2	
F 181	2	6a	1a, 1c, 1e	O6 H1	
F 27	2	7a, 7b	1a, 1d, 1e, 1f	O7 H1	
F 73	2	10a	3a, 3b	O10 H3	
F 219	2	13a	4a, 4c, 4d	O13 H4	
F 485	2	16a, 16b	14a	O16 H14	
F 431	2	23a, 23c, 23d	2a, 2b, 2e, 2f	O23 H2	
F 90	2	24a	4a, 4b, 4e	O24 H4	
F 458	2	26a, 3b	3a .	O26 H3	
F 87	2	28a	3a, 3b .	O28 H3	
F 10	2	29a	13a	O29 H13	
F 384	2	30a	1a, 1c, 1e	O30 H1	

* Subgenus 1 = *P vulgaris*, 2 = *P, mirabilis*

37° C The growth was harvested and suspended in sterile saline These stock suspensions were adjusted to correspond to 50 mg of acetone dried bacteria per ml and were used for preparation of antigens as follows *Veronal buffer extracts* (VE antigen), *freeze press extracts* and *heat extracts* were obtained as described by *Holmgren et al* (11) For some experiments, VE antigens were also boiled for 2½ hours and some VE antigens were treated with trypsin as described by *Holm & Kaijser* (8) *For ultrasonic extracts*, the stock suspensions were treated for 20 minutes at 100 W and 20 Kcs in an MSE ultrasonic Disintegrator 100 W Model (MSE Ltd, London) For each strain, the supernatant after centrifugation at 2000 × *g* for 20 min was used For preparation of *Mickle cell disintegrated antigens* the stock suspensions were mixed with an equal volume of aq dest and 20 per cent (v/v) of aluminium oxide was added The mixtures were treated for 30 minutes in a Mickle Tissue Disintegrator (The Mickle Lab Engineering Co, England) For each strain, the supernatant after centrifugation at 2000 × g for 20 min was used

Purified Antigen Preparations Used for Immunodiffusion Analyses

Lipopolysacharides (LPS) were prepared from the *Proteus* strains O3 H1, O3 H2 and O6 H1 according to *Orskov et al* (27) and were used in concentrations of 1 4 mg ml *Flagella preparations*, were made from the strains O3 H1 and O6 H1 by cultivation on 0 75 per cent nutrient agar plates for 5 days at 15° C and harvesting in sterile saline (37). The suspensions were shaken for 45 minutes Centrifugations were performed at 5000 × *g* three times for 20 min The sediments were discarded The supernatants were centrifuged at 23000 × *g* for 20 min, three times The sediments were saved and the supernatants were centrifuged at 40000 × *g* for 20 min three times

All the sediments obtained after centrifugation at 23000 × *g* and 40000 × *g* as well as the supernatants obtained after the centrifugations at 40000 × *g* were studied by electron microscopy (Electron microscope JEM-T7, Japan Electronics Laboratory Co, Ltd, Japan) The flagella were negatively stained with KPT according to *Horne* (13) The sediments at 23000 × *g* were shown to contain the majority of the flagella with no visible impurities (Fig 1) These preparations were used in the antigen studies

Antigen Preparations Used for Immunization

As regards five of the strains, two types of antigen preparations were used for immunization formalinized or boiled bacterial cultures and for the remaining 10 strains only boiled bacteria were used The bacteria were cultivated in an antigen free medium (7) at 37° C for 6-8 hours The density corresponded to about 2×10^9 bacteria/ml as determined by viable counts By adding formalin to a final concentration of 0 5 per cent the formalinized antigen preparation was obtained Boiled cultures were, after heating to 100° C for 2½ hours washed three times to eliminate the H antigen (18) The sediment was diluted to the original volume and used as antigen In a few experiments freeze-pressed and veronal buffer extracted antigens obtained

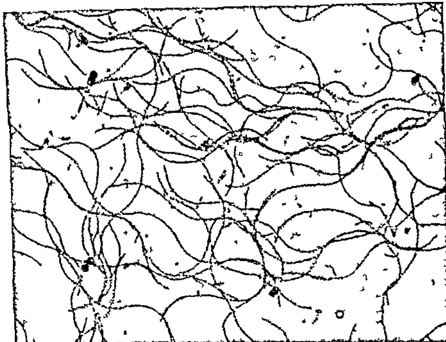


Fig 1 Electronmicrograph of O6 H1 flagella negatively stained with KPT Sediment after centrifugation at 23000 × g Magnification × 15000

from the model strain O3 H1 were mixed in equal parts and used for immunization of rabbits

Preparations of Antisera

Rabbits weighing 2 3 kg were injected intravenously at intervals of 4 8 days with 0 25 05 1 0 1 5 and 2 0 ml with either of the bacterial antigens described above At least two rabbits were used for each antigen preparation The rabbits were bled before every injection and the sera were tested by the indirect haemagglutination method (10) The reciprocal of the titres (½ dilution series) were recorded If the titres did not exceed 4000 the immunizations were continued The animals were sacrificed by cardiac puncture on the 10th day after the last injection By immunization with the formalin killed bacteria OH antisera were obtained The O antisera were produced in rabbits injected with boiled bacterial cultures

O and H antisera against the O3 H1 strain and O antiserum against the O3 H2 strain were kindly supplied by Dr *Beate Perch* Copenhagen and were used in immunodiffusion studies These sera were however 25 years old and had not been tested for activity for many years.

Antiserum to the common protein antigen (cpa) was obtained by hyperimmunization of rabbits with the antigen isolated from an *E coli* strain (16) Antiserum against *E coli* O6 K13 H1 was prepared as described by *Holmgren et al* (11)

Immunodiffusion Methods

In the immunological analyses the following techniques were used

Double diffusion in gel technique as described by *Wadsworth* (35)

Immunoelectrophoresis technique as described by *Wadsworth & Hanson* (36) Separations were made at 5V/cm for 60 90 minutes using an 0 075 M veronal buffer pH 8 6

Crossed immunoelectrophoresis as initially described by *Laurell* (22) was performed in 1 per cent agarose gel The first step at 5V/cm was run for 60 80 minutes and the second step also at 5V/cm for 3 hours The antisera included in the agar were diluted 1/25 and 0 075 M veronal buffer pH 8 6 was used throughout

RESULTS

Comparison of Crude Antigen Preparations from the P mirabilis O3 H1, Strain

When antisera obtained by immunization with a mixture of *P mirabilis* O3 H1 VE and freeze pressed antigens were used with their corresponding antigens, as many as 40 precipitates could be demonstrated by the crossed

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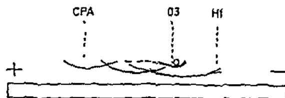


Fig 2 Immunoelectrophoretic analysis of O3 *H1* VE antigen (round basin) reacting with its homologous OH antiserum (long basin) The double contour of the O3 precipitate is shown A similar pattern of the O3 line is obtained with the purified lipopolysaccharide

immunoelectrophoresis technique Using the homologous OH antisera prepared against the formalin-killed bacteria only 6-7 *precipitates* were obtained, using the same technique Using double diffusion and *immunoelectro*phoresis, similar differences between the antisera could be shown These techniques revealed, however, fewer precipitates, i e by immunoelectrophoresis 20 30 and by double diffusion 15 20 precipitates at maximum Comparison of different crude antigen preparations showed the VE antigen as the most complete one, giving the greatest number of precipitates

Identification of Antigens in the P mirabilis *O3 H1, Strain*

The O antigen Using the O3 LPS preparations with the corresponding O as well as *OH* antisera, two precipitates were formed with the double diffusion technique These antigens were heat stable Using immuno-

Fig 3 Immunoelectrophoretic analysis of O3 H1 VE antigen (start basins) reacting with the homologous OH antiserum (long basins) In the cathodic round basins an O6 H1 flagella preparation was placed after the electrophoretic *run.* The line of interference is clearly shown
The O3 H1 VE preparation reacting with its homologous OH antiserum on top can be compared with the schematic Figure 2

IV

III

II

I

CPA

H1

O3

2

1

Fig 4 Schematic drawing of crossed immunoelectrophoresis of *P mirabilis* antigens First and second electrophoretic runs are indicated (1 and 2) Upper round basin H1 flagella preparation Lower round basin O3 H1 VE antigen The plate was divided into four areas I and III were poured with agarose only whereas in areas II and IV the agarose was supplemented with O3 H1 antiserum The identified H1 precipitate is indicated The dotted portion, extending from the H1 precipitate, indicates the form of the precipitate if no additonal basin for the comparative H1 analysis was present

electrophoresis a broad fuzzy precipitate was obtained, the localization of which indicated a rather low electrophoretic mobility of the antigen Occasionally this precipitate split up in the ends, giving two lines (Fig 2) The treatment of the O3 H1 VE antigen with trypsin or boiling did not change the appearance of the O antigen precipitate

The same immunodiffusion results were obtained if the antisera supplied by *Dr Perch* were used

The H antigen The flagella preparations gave in double diffusion analyses one and sometimes two faint precipitates Only one precipitate could be demonstrated by the immunoelectrophoresis techniques used

A *comparison of strains with different O* antigens but all with H1 made it possible to identify this A combination of immunoelectrophoresis and double diffusion was also used to identify H1 (Fig 3) In crossed immunoelectrophoresis the position of the H antigen was determined by comparing a flagellar preparation and an O3 H1 VE preparation diffusing from basins 10 mm apart The flagellar antigen gave a line of identity with one of the precipitates formed by the VE antigens (Fig 4)

The K antigen No antigens with the characteristics of K antigens as shown for *E coli* were found (18, 28)

The common protein antigen (cpa) This antigen described in *E coli* as well as in some strains of *Pseudomonas* and *Neisseria* (16) could be demonstrated in the *Proteus* O3 H1 strain with an *E coli* O6 K13 H1 antiserum or a specific cpa antiserum Using these antisera, the *Proteus* O3 H1 VE antigen formed in immunoelectrophoresis a precipitate indicating a high electrophoretic mobility of the cpa antigen (Fig 5)

Identification of the O Antigens in Different Proteus *Strains*

From the standard strains O3 H1, O6 H1, O10 H3, O13 H4, O16 H14 , O23 H2, O24 H4, O26 H3, O28 H3, O29 H13 and O30 H1, VE antigens were prepared and divided into portions that were boiled or trypsin treated These antigen preparations were each tested against the homologous O antisera in immunoelectrophoresis Their O antigens all formed a broad fuzzy precipitate extending anodically from the start basin, similar to the pattern shown in Fig 3 A total

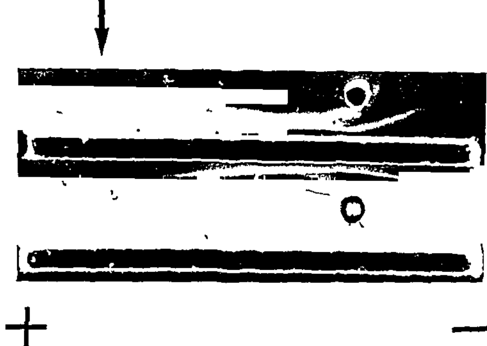


Fig 5 Immunoelectrophoretic analysis of VE antigen from *P mirabilis* O3 H1 (round basins) using homologous OH antiserum (upper long basin) and antiserum to the common protein antigen (lower long basin) The cpa precipitate can be seen to the far left (arrow)

of 49 strains isolated from faeces, blood or the urinary tracts and previously grouped into the 11 O groups mentioned above were compared The immunoelectrophoretic patterns obtained with strains of the same O group were very similar The differences between precipitates formed by different O antigens were rather limited, mainly consisting of variations in the length of the precipitates on the anodic side of the antigen basin

DISCUSSION

Various immunodiffusion techniques have proven advantageous for a characterization of antigens of microorganisms (3 6 9 11, 17) As a basis for the present study on *Proteus* antigens the serological system applying to *Proteus* described by *Perch* (29) has been used This system including *P mirabilis* and *P vulgaris*, contains 49 O groups and 19 H groups, knowledge of the O and H antigens being more detailed for the first 30 O groups The model strain in the present work is thus designated O3a, 3b H 1a, 1c, 1e

In immunodiffusion studies of *E coli* O antigens these antigens have often been found to give two parallel precipitates indicating a heterogeneity of the O antigens Our present observations on *Proteus* O3 antigens points to a similar heterogeneity However, the partial antigenic relationship between 3a and 3b as described by *Perch* (29) could not be discerned by immunodiffusion not even if her original antisera were used in this technique

The flagella preparations made from the strains O3 H1 and O6 H1 gave one distinct precipitate and sometimes an additional faint one whether the homologous or heterologous antisera were used The distinct precipitate [illegible] electrophoretic mobi[illegible] [illegible]hniques [illegible] antigen into 1a, 1c and 1e as described by *Perch* (29) However, her findings were based on direct bacterial agglutination

Studies of *E coli* bacteria have shown that K antigens, capsular antigens, are of importance for the virulence of the bacteria also in relation to UTI (4, 17, 33, 34) Thus it may be of interest to try to detect and analyse such antigens in *Proteus* bacteria as well Although *Namioka et al* (26), for instance have shown the presence of a K(C) antigen in a few *Proteus* and *Rettgerella* strains, no K antigen system has been set up in the case of *Proteus* So far, however, no studies, including the present one, have shown a regular presence of K antigens in *Proteus mirabilis* and *vulgaris*

Cross reactions between strains within the *Enterobacteriaceae* as shown by direct bacterial agglutination or immunodiffusion are well known (11, 34) Also cross reactions with antigens from strains outside the *Enterobacteriaceae*, i e *H influenzae*, *N meningitidis*, have been described (5, 30) A possible protective function of antibodies to these cross reacting antigens has been proposed (31)

In the present study in which VE antigen and the homologous OH antiserum were used, at least some 4 5 antigens were found to be common to most of the investigated *Proteus* strains One of these 4 5 antigens was identified as the common protein antigen (cpa) which has also been found in bacteria from other families such as *Pseudomonadaceae* and *Neisseriaceae* (16) So far the significance of the cross reacting antigens of *Proteus* is not known

The characterization of *Proteus* antigens is of interest for at least two reasons Firstly, they are necessary to allow further epidemiological and oecological studies of the *Proteus* strains causing infections The present investigation has shown that it is possible by immunodiffusion to identify both O and H antigens in strains originally defined by direct agglutination by *Perch* Since according to *Ørskov et al* (28) the future typing of *E coli* possibly will be based on immunodiffusion, this technique should also be applicable to *Proteus* typing Secondly studies of the host-parasite relationship in UTI caused by *Proteus* strains should include analyses of any appearing antibody response Judged from corresponding investigations of UTI caused by *E coli*, O or H antigens should be used as purified and well characterized antigenic preparations Most studies of antibody against *E coli* bacteria have been devoted to the O antibodies (1, 2, 11, 12, 15), but since the H antigen usually seems to be more developed in *Proteus* than in *E coli* bacteria, the H antibodies might be of greater significance in studies of *Proteus* infections Furthermore, testing of H antibodies should be much easier than testing of O antibodies since some 80 per cent of *Proteus* strains investigated by *Kauffmann & Perch* (18) were shown to contain one of the three H1, H2 or H3 antigens, whereas we in preliminary studies have found at least 15 different O groups among *Proteus* strains causing UTI (21)

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EXPERIMENTAL *ASCARIS SUUM* INFECTION IN PIGLETS

Inverse relationship between the numbers of inoculated eggs and the numbers of worms established in the intestine

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Piglets from 0 50 days of age were exposed to infective *Ascaris suum* eggs at dose levels varying from 50 10 000 eggs A clear inverse relationship was found between the inoculated egg doses and the numbers of worms found in the intestine Thus piglets given doses of 1,000 5 000 or 10 000 *A suum* eggs practically never acquired the infection the mean percentage of worms established being 0 013 per cent of the administered egg dose In contrast, when 500 eggs were inoculated, 1 4 5 4 per cent (mean 2 9 per cent) of the egg dose was established, and with a dose of 50 eggs 38 100 per cent (mean 64 per cent) of the inoculated eggs completed their migration and established an intestinal worm population Newborn piglets inoculated before they had suckled colostrum inhibited the infection as effectively as did older pigs On the other hand neonatal piglets did not show the distinct eosinophilia that developed at day 10 in older piglets receiving more than 500 eggs The findings are dicussed with particular emphasis on the mechanisms involved in the massive elimination of migrating *A suum* larvae following inoculation of large egg doses Also the epidemiological consequences of the reported findings are mentioned

Ascariasis in swine is extremely common and most piglets acquire the infection at an early age In contrast it has proved difficult to establish a patent *Ascaris suum* infection by inoculation of infective eggs In studies of migration immunity and pathological lesions of the liver and lungs of pigs or other hosts very large inocula—several thousand eggs—have usually been employed (*Schwartz* 1959 *Roneus* 1966 *Taffs* 1968, *Bindseil* 1969 a b *Douvres et al* 1969) The intestinal worm burden established from such large doses of eggs is strikingly small (*Stewart* 1916 *Green & Oldham* 1964, *Taffs* 1968) *Clapham* (1936) presented some evidence to suggest that an experimental infection in pigs could be obtained more easily if comparatively small doses of eggs were given Recently experimental support of this theory has been reported by *Roneus* (1971) who inoculated eight 7-week-old pigs with doses varying from 10 to 100,000 *A suum* eggs and found that only small doses of 50, 100 and 500 eggs would result in an adult worm population in

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the small intestine, whereas pigs inoculated with 1,000, 10,000 and 50,000 eggs did not establish a patent infection Only one pig was inoculated with each of the doses examined

The available evidence would thus seem to indicate that a study of the relationship between the size of the inoculated egg dose and the numbers of ascarides developing to patency in the gut could be rewarding Since it has been suggested that piglets are especially susceptible to infection with *A suum* during the early neonatal period (*Soulsby* 1965) the experiments to be described below have been performed with different age groups including newborn piglets before they had suckled colostrum

MATERIALS AND METHODS

Two experiments were performed with 38 piglets representing four litters born by two sows (No 64 and 66) of Danish Landrace The sows were purchased during mid pregnancy Coprological examination revealed that one—No 64—excreted small numbers of *A suum* eggs in the faeces Both sows were treated with a thiabendazole/piperazine mixture four times with intervals of one week, the last treatment being given approximately one week before farrowing on which occasion the sows were thoroughly washed and moved to cleaned and desinfected pens in an isolation unit where pigs had not been housed for several months (experiment 1) or for more than one year (experiment 2)

Experiment no 1 The two sows farrowed 9 and 6 piglets, respectively The interval between farrowings was 2 days At three days of age 6 piglets (group A) were inoculated each with 1,000 infective *A suum* eggs The inocula were given orally by means of a syringe fitted with a soft plastic cannula One piglet was kept as a non inoculated control The piglets were left with the sow and sacrificed 30, 34 and 45 days after inoculation (p i), cf Table 1

The remaining 8 piglets (group B) were weaned at 6 weeks One week later, 6 piglets were inocu

cell count was taken from piglets of group B at day 11 p i

Experiment no 2 The next two litters of the sows were born 11 days apart and consisted of 11 and 12 piglets, respectively Two of the 23 piglets were kept as non inoculated controls Three piglets were inoculated directly into the jejunum to examine whether the infectivity of eggs introduced by this route would be different from eggs inoculated orally into three other piglets of the same age *Ascaris* eggs, cultured in the laboratory, lack an albuminous layer which possibly render them more suspectible to hydrochloric acid of the stomach Age groups and doses in experiment 2 are listed below

At birth	Six piglets were given 5 000 eggs orally before they had suckled colostrum They were returned to the sow 4 hours later
At 4 days	Three piglets received 5,000 eggs orally Three piglets received 5 000 eggs injected into the mid jejunum after laparotomy under universal anaesthesia
At 28 days	Three pigs received 50 eggs orally Three pigs received 500 eggs orally Three pigs received 5,000 eggs orally

Piglets were sacrificed 38 77 days p i

Preparation of infective A suum *eggs* Adult specimens of *A suum* were obtained from a slaughterhouse Eggs were removed from the uteri through a longitudinal incision and examined under the microscope Batches of fertile eggs were incubated in tap water with 1 per cent formalin The cultures were kept at 24 degrees Centrigrade in large Petri dishes and agitated slightly each day Infective eggs developed in appr 28 days The cultures used for inoculation contained appr 70 per cent infective eggs as judged from morphological criteria The culture used in experiment no 2 was appr 5 months old which did not appear to affect the infectivity Small inocula of 50 eggs were counted under the microscope Larger inocula (500, 1 000, 5 000 and 10 000 eggs) were measured by volume after determination of the number of eggs in the stock culture by means of a McMaster counting chamber

Clinical and haematological procedure The piglets were observed daily They were agitated to facilitate the detection of such signs as coughing and dyspnoea Body temperatures were not recorded All piglets received an intramuscular injection of iron-dextran (150 mg) and vitamin A (50 000 I U) during the first week of life In experiment no 2 eosinophil counts were determined twice weekly Blood was sucked from an ear vein directly into a heparinized pipette Total eosinophil counts (eosinophils/mm³) were determined in a Fuchs Rosenthal counting chamber Differential white cell counts were done on blood smears stained with May-Grunwald-Giemsa stain

Post mortem procedure The intestine was removed immediately after death and separated from

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Piglets from 0 50 days of age were exposed to infective *Ascaris suum* eggs at dose levels varying from 50 10 000 eggs A clear inverse relationship was found between the inoculated egg doses and the numbers of worms found in the intestine Thus piglets given doses of 1,000 5 000 or 10,000 *A suum* eggs practically never acquired the infection the mean percentage of worms established being 0 013 per cent of the administered egg dose In contrast, when 500 eggs were inoculated, 1 4 5 4 per cent (mean 2 9 per cent) of the egg dose was established, and with a dose of 50 eggs, 38 100 per cent (mean 64 per cent) of the inoculated eggs completed their migration and established an intestinal worm population Newborn piglets inoculated before they had suckled colostrum inhibited the infection as effectively as did older pigs On the other hand neonatal piglets did not show the distinct eosinophilia that developed at day 10 in older piglets receiving more than 500 eggs The findings are dicussed with particular emphasis on the mechanisms involved in the massive elimination of migrating *A suum* larvae following inoculation of large egg doses Also the epidemiological consequences of the reported findings are mentioned

Ascariasis in swine is extremely common and most piglets acquire the infection at an early age In contrast, it has proved difficult to establish a patent *Ascaris suum* infection by inoculation of infective eggs In studies of migration, immunity and pathological lesions of the liver and lungs of pigs or other hosts very large inocula—several thousand eggs—have usually been employed (*Schwartz* 1959 *Roneus* 1966, *Taffs* 1968, *Bindseil* 1969 a b, *Douvres et al* 1969) The intestinal worm burden established from such large doses of eggs is strikingly small (*Stewart* 1916, *Green & Oldham* 1964, *Taffs* 1968) *Clapham* (1936) presented some evidence to suggest that an experimental infection in pigs could be obtained more easily if comparatively small doses of eggs were given Recently experimental support of this theory has been reported by *Roneus* (1971) who inoculated eight 7-week-old pigs with doses varying from 10 to 100,000 *A suum* eggs and found that only small doses of 50, 100 and 500 eggs would result in an adult worm population in

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TABLE 2 *Data from Experiment No 2*

Pig	Born	Sex†	Inoculation		Eosinophils§		Post mortem		
			Age at inoculation (days)	Number of eggs inoculated	Maximum counts	Day p i	Days after inoculation	Number of worms in gut	Number of milk spots*
1	15/2	f	0	5 000	182	46	76	5	0
2	15/2	f	0	5 000	378	53	56	0	2
3	15/2	m	0	5,000	181	60	72	0	2
13	26/2	m	0	5 000	575	8	70	2	0
14	26/2	f	0	5,000	560	11	74	1	0
15	26/2	f	0	5 000	344	15	77	0	0
5	15/2	m	4	5 000$	181	28	68	0	1
6	15/2	f	4	5 000$	300	10	72	0	0
12	15/2	f	4	5,000$	450	10	52	0	0
7	15/2	m	4	5 000	388	56	72	0	0
8	15/2	f	4	5 000	600	49	52	0	1
9	15/2	m	4	5,000	825	10	72	0	0
16	26/2	f	28	50	325	4	38	50	0
17	26/2	f	28	50	325	22	42	27	0
18	26/2	m	28	50	1070	14	45	19	0
19	26/2	f	28	500	2250	10	38	27	3
20	26/2	f	28	500	1460	10	42	7	2
21	26/2	m	28	500	1740	10	45	9	4
22	26/2	m	28	5,000	1480	10	38	1	0
23	26/2	f	28	5 000	1740	10	42	0	3
24	26/2	f	28	5,000	845	10	45	1	2
4	15/2	m	Not inoculated		144		*	0	2
11	15/2	f	Not inoculated		850		*	0	0

† f = female, m = male

§ Eosinophils/mm³

* Control pigs 4 and 11 were sacrificed at 72 days of age.

$ Pigs 5, 6 and 12 were inoculated into the small intestine All other inoculations in the table are by the oral route

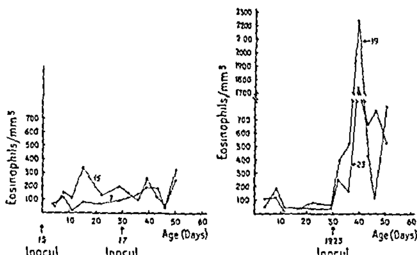


Fig 1 Eosinophil counts in four piglets, inoculated with *A suum* eggs at times indicated by arrows.
Piglet no 15 5,000 eggs immediately after birth
Piglet no 17 50 eggs at 4 weeks of age
Piglet no 19 500 eggs at 4 weeks of age
Piglet no 23 5,000 eggs at 4 weeks of age

However, by far the greatest worm burden was established in the three piglets that received 50 eggs Nineteen, 27, and 50 *A suum* were recovered from the intestine of these piglets, i e 38-100 per cent (mean 64 per cent) of the administered dose

There was no significant difference in numbers or severity of liver lesions ("milk spots") among piglets receiving low and high doses of *A suum* eggs (Tables 1 and 2) In all groups "milk spots" occurred in low numbers

Eosinophil counts (Fig 1) Piglets inoculated with 5 000 eggs *at birth* had no distinct rise in eosinophil counts However, the same administered to 4-week old piglets caused a marked eosinophilia with maximal counts at day 10 p i Individual maxima varied from 845 to 1 740 eosinophils/mm³ Corresponding relative values were 17 34 per cent eosinophils of 200 counted leucocytes in the blood smear A similar or even more marked eosinophilia occurred in the piglets receiving 500 eggs at 4 weeks of age (Fig 1 Table 2)

Only one of the three piglets receiving 50 eggs at 4 weeks showed a distinct eosinophil response, with a maximum level of 1,070 eosinophils/mm³ at day 14 p i (pig no 18, cf Table 2)

DISCUSSION

The present data give strong support to *Roneus* (1971) who appears to be the first to suggest an inverse relationship between size of the inoculated egg dose and numbers of ascarides established in the intestine When the two experiments reported above are considered together it will appear that with doses above 1,000 eggs infection practically never occurred Thus, in experiment no 1 only one ascarid worm was recovered from two groups of 6 piglets inoculated at three days and at 7 weeks with 1,000 and 10,000 eggs, respectively Likewise, in experiment no 2, 10 out of 15 piglets receiving 5,000 eggs at birth, at 4 days (orally or injected into the intestine) or at 4 weeks did not become infected and the remaining 5 piglets in this experiment harboured extremely small worm populations of 1-5 specimens in the intestine Piglets inoculated during the early neonatal period appeared to inhibit the infection as effectively as did older piglets (Table 1 and 2)

In contrast, piglets receiving a dose of 500 infective eggs at 4 weeks of age established worm burdens of 1 4 5 4 per cent (mean 2 9 per cent) of the inoculated dose, and with

a small dose of 50 eggs, 38-100 per cent (mean 64 per cent) became established in the gut

This train of events appeared to be reflected in the eosinophil counts Piglets receiving doses of 500 or 5,000 eggs at four weeks had a marked eosinophilia with maximum levels at day 10 p i, whereas the small dose of 50 eggs was not associated with nearly the same degree of eosinophil response Here, it should also be mentioned that a high dose of 5 000 eggs given to 0-3 day old piglets did not provoke an eosinophilia (Figure 1)

The mechanisms involved in the very efficient elimination of migrating larvae from large inocula are virtually unknown Nor is the site of the elimination known *Schwartz* (1959) noticed that pigs given 10 000 *A suum* eggs eliminated large numbers of immature worms via the stools from the fourth week p i and onwards The same phenomenon has been described by *Douvres et al* (1969) who stated that large numbers of 4th stage larvae were expelled from the gut, beginning 10 days after inoculation of 100,000 *Ascaris* eggs *Taffs* (1968) considers the liver to be an important site for destruction of migrating larvae *Bindseil* (1970) on the other hand, states that the liver is not involved in the defence mechanisms of immune mice challenged with *A suum* eggs and suggests that the protective immunity is exerted in the lumen of the intestine by inhibition of hatching of the inoculated eggs and, possibly, by inhibition of penetration of the intestinal mucosa Further studies are required to determine whether the very efficient elimination of a large egg dose is exerted in the liver, the gut or elsewhere The extent and severity of liver lesions in piglets studied in the present work were not suggestive of a massive cellular reaction Numbers of ' milk spots ' were very low, irrespective of the dose inoculated

It is tempting to suggest that the marked eosinophil response reflects an immune reaction towards migrating larvae One function of the eosinophil granulocyte is to phagocytize immune complexes and the time of maximal peripheral eosinophilia found here (10 days p i) coincides with the appearance of circulating antibodies against *A suum* reported by *Taffs* (1968) Likewise, in mice infected with 10,000-12,000 *A suum* eggs, there was a distinct rise in serum IgM with maximum levels 8 days p i (*Crandall* & *Crandall* 1971) In guinea pigs there is a progressive accumulation of eosinophils in the lungs during the first week following initial infection with 50,000 100,000 hatched second stage larvae injected into the portal vein (*Soulsby* & *Muncey* 1970) On the other hand, although reflecting immune reactions, the eosinophil granulocyte is probably not *primarily* involved in the defence mechanisms against migrating *A suum* larvae This may be tentatively concluded from the finding that piglets inoculated with 1,000 and 5,000 *A suum* eggs during the early neonatal period (0 3 days of age) inhibited the infection as effectively as did older piglets although they did not respond with a peripheral eosinophilia (cf Figure 1) Similar findings were reported in experimental *Fasciola hepatica* infection in calves with a congenital defect in their cellular immune system (genetic code A46) When such calves were inoculated with metacercariae they did not respond with a local or a peripheral eosinophilia but nevertheless, they inhibited the infection as effectively as did normal calves in which the migration of larvae through the liver was associated with a distinct eosinophil response (*Flagstad et al* 1972) Obviously the function of the eosinophil granulocyte in parasitic infections needs further study

The results obtained in the *present work* would indicate that earlier studies of ascariasis in pigs should be reconsidered Thus, investigations of the pathogenesis of the *infection*, using very large inocula -up to 100,000 *eggs*, are probably not relevant to the situation to which pigs are exposed in nature although they could be useful to determine the cause and site of the massive elimination of the infection that follows

Several surveys have shown that adult swine carry very low *A suum* burdens. E g

Jacobs (1967) found an average of three *Ascaris* worms per sow in a *post mortem* study of Danish pigs Moreover, a large proportion of the *A. suum* eggs passed by sows are non-fertile As a consequence, the validity of the widely employed anthelmintic treatment of sows before farrowing has been questioned The data presented by *Roneus* (1971) and the authors, however, would permit the conclusion that sows passing few *Ascaris* eggs via the faeces are, in fact, particularly liable to induce patent infections in their offspring and, consequently, a complete elimination of the worm by routine medical treatment of pregnant sows should be aimed at, although this may prove very difficult to achieve

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LOCALIZATION OF HOST ANTIGENS IN THE EGG-GROWN INFLUENZA VIRUS PARTICLE

II Demonstration of the Forssmann Hapten in the Lipid Layer and the 'Host Antigen' on the Viral Spikes

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Purified egg grown influenza virus A/PR/8/34 (HON1) was shown to contain the Forssman hapten in the lipid fraction. Forssman determinants could not be demonstrated in the influenza proteins The strain X 31 (H3N2) was also shown to have the Forssman hapten The keratosulphatelike host antigen seemed to be located on both types of spikes

We have earlier shown that purified egg grown influenza virus A/PR/8/34 (HON1) does not combine with antibodies to the Forssman (F) antigen (10), in contrast to findings by others (5 6 16) The critical purification step was found to be isopycnic banding in CsCl By this method the virions were separated from membrane like fragments which had the capacity of agglutinating erythrocytes and reacting with antibodies to the F antigen

Our investigations did not exclude the presence of F determinants in deeper layers of the virion preferably in the lipid layer since the F hapten is known to be a glycolipid (1, 14 19)

In the present work we have examined the viral lipids serologically in two ways, by testing the extracted lipids in haemolysis inhibition tests and by examining the spike devoided influenza particles for absorption of antibodies to the F antigen

Laver & Webster (12) showed that the keratosulphatelike host antigen was located on the haemagglutinin spikes examining an A0- and a B strain of influenza virus The second part of the present study concerns attempts to see if the neuraminidase spikes possess this antigen as well

MATERIALS AND METHODS

Virus The A/PR/8/34 (HON1) and the recombinant X 31 (H3N2) strains were grown in the allantoic cavity of 10 day chicken embryos The latter strain is a recombinant between the Hong Kong strain A/Aichi/68 (H3N2) and the above mentioned A0-strain The recombinant kindly provided by Dr *G C Schild* World Influenza Centre, Mill Hill London possesses both the haemagglutinin and neuraminidase from the Hong Kong parent while the high growth potential in eggs is retained from the A0 strain (11) The strains are given the shorthand notations A0/PR8 and X 31 respectively

Virus purification Both strains were purified as described earlier (10), but adsorption and elution

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were performed with bovine erythrocytes for the recombinant in accordance with *Laver & Webster* (13)

Antisera The anti F serum (R 745) and the anti B/Lee sera (R 85 and R 86) were produced in rabbits (10) The rabbit anti X 31 serum was produced by repeated intravenous injections

Serological tests Virus haemagglutination (HA) and *haemagglutination inhibition* (HI) tests were done in WHO trays by the standard method with 0 5 per cent chicken erythrocytes or 1 per cent guinea pig erythrocytes *Haemagglutination inhibition blocking* (HIB) was performed according to *Harboe* (8) and *Haukenes et al* (9), and the *haemolysis* (HL) test as described earlier (10) *Haemolysis inhibition* (HLI) tests were done in small tubes by the method of *Makita et al* (14) To 0 25 ml of twofold dilutions of antigen in phosphate buffered saline (PBS), pH 7 2, were added equal volumes of heat inactivated anti F serum, containing 4 haemolytic doses After 60 min at room temperature 0 25 ml of fresh guinea pig serum diluted to contain 4 haemolytic doses of complement, and 0 50 ml of 0 5 per cent sheep erythrocytes was added The degree of lysis was graded visually after incubation at 37° C for 45 min The HLI titre is expressed as the reciprocal value of the highest antigen dilution yielding more than 50 per cent inhibition of lysis To avoid any anti-complementary effect, the antigens were mixed with equal volumes of undiluted guinea pig serum before the HLI assay and incubated at 37° C for 30 min followed by heating at 56° C for 30 min *The neuraminidase inhibition* (NI) test was performed according to *Webster & Pereira* (21) with some adjustments To 0 1 ml of neuraminidase, adjusted to liberate an amount of sialic acid giving an optical density (OD) of 0 5 to 0 7 in the Warren test (20) modified by *Aminoff* (2) were added twofold serial dilutions of heatinactivated antiserum in PBS After incubation for 30 min at 37° C and overnight at 4° C 0 3 ml of fetuin ~ 5 mg/ml in 0 1 M phosphate buffer, pH 5 9) was added to each tube and the mixture was incubated for 60 min at 37° C

The OD was read against the reagent blank (fetuin + saline) Both preimmune and immune sera were tested the NI titre representing the reciprocal value of the highest serum dilution giving 50 per cent inhibition of the enzymatic activity after correction for preimmune readings Attempts to block the neuraminidase inhibition by host antigen were made by the following method To twofold dilutions of the host antigen in PBS was added an equal volume of heat inactivated serum containing 4 NI-doses/0 1 ml After 60 min at room temperature 0 1 ml of each dilution mixture was transferred to another row of tubes and 0 1 ml of enzyme, containing the same activity as in the NI test, was added to each tube After incubation for 60 min at 37° C and overnight at 4° C, 0 3 ml of fetuin was added Liberated sialic acid was quantitated as above

Host antigen This was purified from chick allantoic fluid according to *Haukenes et al* (9) The HIB titre of 1 mg/ml solution was ≧ 32 000

Lipid extraction The lipids were extracted from the purified viral preparations as described by *Folch et al* (7) and *Schultze* (17), and prepared for serological investigations by the method of *Makita et al* (14)

Bromelain treatment Bromelain (EC 3 4 4 24, Sigma) degradation of purified A0/PR8 was done according to *Compans et al* (4) The final bromelain concentration was 1 mg/ml After 4½ h at 37° C the HA titre was below 2, as compared to an unchanged titre of 10 240 in the control virus suspension, incubated without enzyme and SH reagent (0 05 M dithiothreitol, Sigma) The reaction was stopped by pelleting the virus particles in the cold (see below) Thereafter the resuspended pellet material was centrifuged to equilibrium in CsCl (10)

For bromelain treatment of strain X 31 the method of *Bachmayer & Schmidt* (3) was used Purified virus was incubated for 24 h at 4° C with a final bromelain concentration of 6 mg/ml The virus particles were then pelleted and resuspended in 0 1 M phosphate buffer, pH 5 9 while the supernatant was dialyzed in the cold against the same buffer to remove residual SH reagent Neuraminidase activity was measured in these fractions Thereafter a rate zonal centrifugation of the bromelain supernatant was carried out in a 16 ml linear 5–20 per cent (w/v) sucrose gradient (see below) The tubes were punctured at the bottom and fractions of 1 ml each were collected The fractions were dialyzed in the cold against 0 1 M phosphate buffer, pH 5 9, and then assayed for neuraminidase activity A sample of anti F serum was run in parallel in order to locate the positions of 19S and 7S γ globulins The haemolytic activity in the serum fractions was measured in the HL test described above The sedimentation coefficient for the unknown material was determined according to *Martin & Ames* (15)

Ultracentrifugations All centrifugations were done in a Beckman Preparative Ultracentrifuge Spinco $L_2$65B, at 4° C The pelleting of virus particles, bromelain treated or not was done with an SW 50 1 rotor at a speed of 35 000 rpm for 45 min In the absorption experiments the centrifugation time was 30 min Rate zonal centrifugation was done by layering 1 0 ml of material on top of a preformed 16 ml linear 5–20 per cent (w/v) sucrose gradient and centrifuging for 18 h at 27 000 rpm with an SW 27 1 rotor and stabilizer

Electron microscopy Our previously reported method was used (10)

EXPERIMENTS AND RESULTS

Forssman activity of Spikeless A_O/PR8 virus

As described in Methods the bromelain treated A_O/PR8 virus was re purified by centrifuging to equilibrium in CsCl The bromelain treated and control virus banded at densities of 1 17 g/cm³ and 1 22 g/cm³, respectively Electron microscopic examination of the respective preparations revealed "naked , bean shaped and spikeless particles versus normal virions (Figures 2 and 1)

Both preparations were tested for capacity *to bind antibodies to the F antigen To 50 µg* of material obtained from the two density positions of the bromelain- and control tubes, was added 5 ml of heat inactivated anti F serum diluted 1 2,000 in *PBS, i e 16 HL doses/0 25 ml After incubation for 30 min* at 37° C the tubes were cooled and centrifuged as stated in Methods Haemolytic activity was measured in the supernatants, the results being given in Table 1 It is seen that the spikeless particles, in contrast to the normal virions, absorb antibodies to the F antigen This was confirmed by examining the viral preparations incubated with anti F serum and complement, in the electron microscope Only spike devoided particles had the characteristic halo of γ globulins/complement complexes (Figure 4) The intact virions looked completely normal after the same *treatment As a control anti B/Lee serum* (*i e* anti host) was tested instead of anti F



Fig 1 Normal virus particle with density 1 22 g/cm³ Bar 1000 Å



Fig 2 Spike less A_O/PR8 particles with density 1 17 g/cm³ Bar 1000 Å

TABLE 1 *Absorption of Antibodies to the Forssman Antigen with Preparations of Normal and Bromelain Treated A_O/PR8*

Virus preparation	Density g/cm³*	HA titre	HL titre before/ after absorption × 10³	Activity remaining %
Bromelain treated	1 17	< 2	32/2	6
	1 22	< 2	32/32	100
Control	1 17	< 2	32/32	100
	1 22	10 240	32/32	100

* After bromelain treatment viral cores *i e* spikeless particles and intact virions banded at densities of 1 17 g/cm³ and 1 22 g/cm³ respectively upon isopycnic banding in a CsCl gradient

42*



Fig 3 A_O/PR8 + heat inactivated and periodate treated anti B/Lee serum + fresh guinea pig serum The virus particles show 'haloes' of bound complexes of antibodies and complement Bar 1000 A



Fig 4 Spike less A_O/PR8 particle + heat inactivated and periodate treated anti F serum + fresh guinea pig serum The virus particle shows a hal of bound complexes of antibodies and complement Bar 1000 A

serum Here only intact virions did bind antibodies and complement (Figure 3)

Forssman activity of the A_O/PR8 Lipids and Proteins

The lipids extracted from purified A_O/PR8 virus, were prepared for serological investigation and saturated with fresh guinea pig serum as described in Methods The final volume of the lipid emulsion was 1 ml The aqueous phase of the lipid extraction mixture was concentrated by vacuum dialysis against PBS and the volume adjusted to 1 ml

The supernatant after pelleting the bromelain treated particles was concentrated in the same way from 5 to 2 ml This concentrate was tested for F-activity in the HLI assay together with the influenza lipids and the water-soluble fraction The results are given in Table 2 No F-activity could be detected in the aqueous phase, indicating the lack of F-determinants of the viral proteins The viral lipids, however, showed definite HLI activity, indicating the presence of the F-hapten

TABLE 2 *Haemolysis Inhibition Tests with Ao/PR8 Lipids and Supernatant after Pelleting of Bromelain Treated Virus*

Preparation		HA titre before treatment	HLI titre
Lipid extract	Organic phase	8×10^1	32
	Aqueous phase		< 2
Concentrated bromelain supernatant		5×10^4	< 2

The Forssman Hapten in Influenza Virus X-31

According to *Bachmayer & Schmidt* (3) the bromelain enzyme will split off only the neuraminidase spikes from A_2-viruses at 4° C As seen from Table 3 74 per cent of the

TABLE 3 *Neuraminidase Activity of Bromelain Treated and Control Virus*

Virus preparation	HA in pellet	Neuraminidase in* super natant	Neuraminidase in* pellet
Bromelain treated	512	0 67 (74%)	0 24 (26%)
Control	512	0 04 (8%)	0 55 (92%)

* Expressed as OD at 549 nm in the Warren test

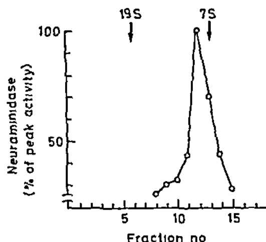


Fig 5 Neuraminidase activity after rate zonal centrifugation of the bromelain supernatant through a 16 ml linear 5 to 20 per cent (w/v) linear sucrose gradient The positions for the 19 S and 7 S γ globulins are ind cated

neuraminidase activity of strain X-31 was found to be liberated, whereas only 8 per cent was found in the control virus super natant No reduction in HA titre was observed in consequence of this treatment Electron microscopically, very few spikes seemed to have been removed by bromelain Thus a significant liberation of HA spikes is not likely It is seen from Table 4 that bromelain-treated virus, in contrast to control virus, was inhibited by anti F antibodies in the HI-test (This inhibition could be blocked by a homogenate of guinea pig kidney) This may be explained by exposure of part of the lipid layer when the neuraminidase (and some HA?) spikes are removed The antibodies bound may consequently sterically hinder the neighbouring HA spikes from reacting with erythrocytes It is also seen from the table that antibodies to the F antigen did not inhibit the neuraminidase activity, irrespective of whether the enzyme was separated from the virions or not

Estimation of Sedimentation Coefficient of the Liberated Neuraminidase

The neuraminidase in the supernatant from the bromelain experiment was purified by centrifuging through a preformed, linear 5 to 20 per cent (w/v) sucrose gradient as described in Methods In Figure 5 the neuraminidase activities are plotted together with the 19S and 7S positions obtained by running in parallel a sample of anti-F serum The calculated S-value is 8 6 ± 0 5 S irrespective of the marker used for calculation The probable limits are set on the basis of the uncertainties in determining the distance to the meniscus Deviations from linearity in the sucrose gradient and any differences in specific volume between the neuraminidase and the γ globulins have not been taken into consideration

Determination of Host Antigen Activity in Normal and Bromelain treated Influenza Virus X-31

The host antigen was quantitated in the HIB test HA active preparations were heated for 5 min at 100° C before assay The heating destroys the neuraminidase and HA-activity of the virus particles, while the serological activity of the host antigen remains intact (8, 9) The blocking titres for various preparations are shown in Table 4 The table

TABLE 4 *Serological Tests with Purified X 31 Neuraminidase, Bromelain Treated and Control Virus*

Virus preparation	Anti serum to	HI titre	HIB-titre	NI titre
Neuraminidase*	B/Lee§		256	< 2
	F			< 2
	X 31			210
Bromelain treated virus	B/Lee	256	32†	
	F	64		
	X 31	1024		
Control virus	B/Lee	64	128†	< 2
	F	8		< 2
	X 31	512		192

* Purified by rate zonal centrifugation through a 5 20 per cent (w/v) linear sucrose gradient
§ Both R 86 and R 85 were tested here, otherwise only R 86
† Neuraminidase and HA activity inactivated by heating for 5 min at 100° C

shows that the blocking activity of the brom elain treated particles is only 1/4 that of intact virions That the explanation of this is the removal of the neuraminidase spikes from the virus particles is supported by the high blocking titre of the purified neuraminidase itself When the neuraminidase was heated for 5 min at 100° C the blocking titre re mained unchanged (not shown in the table)

The possibility of a 'contamination with monovalent HA not separated from the neuraminidase by rate zonal centrifugation could not be excluded The neuraminidase preparation was therefore absorbed with chicken erythrocytes which will remove all HA material but not the neuraminidase 0 25 ml of packed erythrocytes was added to 0 50 ml of the neuraminidase preparation After 30 min in the cold the erythrocytes were spun down and the supernatant tested for enzym atic activity and HIB activity No significant deviations from pre absorption results were obtained showing that the host antigen mate rial of our preparation was not located on contaminating HA spikes

Our results from the HI tests like those earlier reached by others (12) in a different experimental approach show that the host antigen is located on the HA spikes as well anti B/Lee antibodies having strongly in hibited the HA of neuraminidase devoided particles (Table 4)

Inhibition of Neuraminidase Activity by Various Antisera

When antibodies are bound to the neura minidase spikes the enzymatic activity may be inhibited The assay used is the well known neuraminidase inhibition test with the ad justments described in Methods It was found that the enzymatic activity could not be hindered by antibodies to the host antigen since neither of the two anti B/Lee sera used one having an HI titre exceeding 512 (R 86) had a measurable NI titre The homologous antiserum on the other hand strongly in hibited the enzymatic activity (Table 4)

Attempts to block the neuraminidase in hibition by the anti X 31 antibodies with large amounts of purified host antigen (1 mg/ml) were unsuccessful The importance of this finding will be discussed

DISCUSSION

The Forssman Hapten

The results in this and our earlier report (10) indicate that the F hapten is incorpor ated into egg grown influenza virions The fact that the F hapten is a glycolipid (1 14 19) fits very well with our results as the F activity could only be found in the influ enza lipids That the sugar residues of the F hapten are part of the viral glycoproteins does not seem likely Neither the aqueous phase of the lipid extraction mixture nor the con centrated supernatant in the bromelain ex periment reacted in the HLI tests indicating the lack of F activity Reservations must be made however, with regard to a possible proteolytic action on the virus particles by the bromelain enzyme producing small dialyz able fragments which escape during con centration by vacuum dialysis

Furthermore the anti F antibodies did not inhibit the X 31 enzyme (Table 4) and the enzyme did not react in the HLI test (not included in tables) When neuraminidase spikes were split off the X 31 virus anti F antibodies inhibited the virus mediated haemagglutination All these findings point to the conclusion that the F hapten is located cryptically in the virions i e in the lipid layer where it is not accessible to the antibody molecules

The Host Antigen

The investigations concerning the locali zation of the keratosulphate like host antigen were restricted to the recombinant X 31 (H3N2) strain because of the relative ease of selective removal of neuraminidase spikes from this strain using the SH requiring prot ease bromelain The sedimentation coefficient found for the liberated neuraminidase spike 8 6 ± 0 5 S is in accordance with findings by

other workers (18 23) The high HIB titre of the neuraminidase spike preparations strongly indicate that the host antigen is located in the neuraminidase spikes The difference in the blocking titres of heated normal and bromelain treated particles is in accordance with this view as is also the result of an examination of a hyperimmune rabbit anti N2 serum kindly supplied by Dr *G C Schild*, London This antiserum gave a precipitation line in double diffusion with purified host antigen and inhibited egg grown B/Lee virus to a titre of 800 in the HI test (not included in the results) The neuraminidase used for immunization was purified by a procedure different from ours namely by cellulose acetate electrophoresis of SDS treated particles (*G C Schild*, personal communication) Also *Webster* & *Laver* (22) obtained antibody to the host antigen in antisera against neuraminidase subunits isolated by SDS-electrophoresis

The somewhat high blocking activity of the intact neuraminidase molecule may be explained by exposure of more antigenic sites on release from the virion The fact that the host antigen seemed to be located *on the* neuraminidase spike may be expected to be of importance when determination of both antigenic shift and drift is done with egg grown virus and vaccine sera However the NI test with anti B/Lee serum did not give measurable NI titres Homologous antiserum anti X 31 had an inhibition titre of 210 but this effect seemed exclusively to be the result of anti neuraminidase antibodies provoked by the virus coded proteins because attempts to block the neuraminidase inhibition with large amounts of host antigen were unsuccessful The conclusion to be drawn from this is that the host antigen must be located at a considerable distance from the enzymatic site In contrast to the interference in the HI tests by the host antigen on the HA spikes (22) this need not be corrected for in the NI tests Whether this is the case for other influenza strains than the X 31 cannot be assumed *a priori* although it would be a reasonable guess Other substrates than fetuin with other molecular dimensions may also give different results than those presented here Further investigations must be done in order to clarify this

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ELECTRON MICROSCOPY OF *LEPTOSPIRA*

I Leptospira *Strain Pomona*

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Leptospira strain Pomona was studied by negative staining and ultrathin sectioning techniques The effect of various fixatives on the preservation of cellular details was evaluated The helical cytoplasmic cell bodies were surrounded by a triple layered cytoplasmic membrane with an intermediate layer in close apposition to the outer aspect of the membrane The outer envelope of the cell consisted of an ordinary triple layered membrane with an additional layer exterior to it This exterior layer may roll off or break off from damaged leptospira cells, and may then be found as finely striated tubules in negatively stained preparations Usually cells had two axial filaments, one inserted subterminally at either end of the cell The filaments were entwined around the cytoplasmic body and were situated between the cell body and the outer envelope An overlap of the axial filaments on the body of the cell was not observed Dividing cells had four axial filaments, one inserted at either end of the two daughter cells The dimensions and the substructure of liberated axial filaments were studied and compared with those on flagella of treponemes and bacteria

Recent studies carried out with the electron microscope and by other means (3 18, 22) have revealed that leptospires possess four main anatomical features, *viz* 1) a procaryotic cytoplasmic body delimited by a cytoplasmic membrane, 2) a mucopeptide containing layer closely associated with the cytoplasmic membrane and believed to be rigid enough to exert the helical shape on the organism, 3) two axial filaments each inserted in the cytoplasmic body subterminally at opposite ends of the organism, and 4) an outer membrane or sheath enveloping the entire organism

The present paper reports the results of electron microscopical studies on *Leptospira* strain Pomona with particular reference to morphological details of the features mentioned above

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MATERIAL AND METHODS

The *Leptospira* strain Pomona received 1937 from Professor *Schuffner*, Institute of Tropical Hygiene, Amsterdam, was cultured at 29° C in Korthof's medium (16) containing ca. 9 per cent of inactivated rabbit serum and enriched with rabbit haemoglobin and an aqueous extract of baker's yeast (6) Five day old cultures were centrifuged for 30 minutes in a water cooled centrifuge at 15 000 *g* The wet sediment was resuspended in Korthof s basal medium, that is a peptone salt solution, to about 1/6 of the original volume

Preparations for Negative Staining

One volume of the suspension of leptospires in Korthof's basal medium was added to one volume of the fixative chosen The fixatives used were a) 1 per cent or 0 05 per cent (w/v) OsO_4 in either phosphate buffered saline pH 7 3 (PBS) or a salt

solution containing sucrose (SMC) consisting of 0.01 M $MgCl_2$, 0.01 M $CaCl_2$ and 0.03 per cent (w/v) sucrose in distilled water, b) 0.4 per cent (v/v) glutaraldehyde in PBS or SMC, and c) 8 per cent or 4 per cent formaldehyde (19) in PBS or SMC.

The cells were fixed for 30 minutes at room temperature (22–25° C) inclusive centrifugation at 15 000 g for 10 minutes.

Unfixed cells were prepared by adding one volume of PBS or SMC to one volume of the cell suspension prior to centrifugation at 15 000 g for 10 minutes.

The wet pellets of fixed or unfixed cells were suspended in a few drops of PBS or SMC and negatively stained on carbon reinforced Formvar coated grids by a multiple drop technique described earlier (14). The following stains were used: a) 1 per cent (w/v) ammonium molybdate adjusted to pH 7 with NH_4OH, b) 2 per cent (w/v) sodium silicium tungstate adjusted to pH 7 with NaOH, c) 1 per cent (w/v) phosphotungstic acid adjusted to pH 7 with KOH, and d) 1 per cent (w/v) uranyl formate adjusted to pH 6 with NH_4OH.

Axial filaments were liberated by treating cells with the detergents Teepol or sodium deoxycholate, or with the proteolytic enzyme *Myxobacter* AL 1 protease I*. Details of these procedures have been described previously (14).

Preparations for Sectioning

10 ml SMC + 1 ml fixative were added to 10 ml of suspensions of cells in Korthof's basal medium. The fixatives were: a) 1 per cent OsO_4 in SMC, b) 3 per cent glutaraldehyde in redistilled water and c) 8 per cent formaldehyde (19) in SMC. Prefixation time was 30 minutes at room temperature inclusive centrifugation at 15 000 g for 10 minutes. The pellets of cells were enrobed in 45° C warm melted agar (1.5 per cent Noble Agar Difco in SMC). Agar blocks of about 1 mm^3 were cut and fixed overnight at room temperature in 1 per cent OsO_4 in SMC containing 10 per cent YAP medium§. After a brief wash in SMC the blocks were treated for 1 hour at room temperature with 2 per cent uranyl acetate (23) in SMC, then dehydrated in alcohol and propylene oxide (17) and finally embedded in Vestopal W (24).

Sectioning, Post staining and Electron Microscopy

Ultrathin sections were obtained on the LKB ultrotome I and III microtomes. The sections were post stained by floating the grids, section side down on magnesium uranyl acetate (11) and afterwards on lead citrate (21).

Electron microscopy was carried out on Philips EM 200 or 300 electron microscopes at primary magnifications of 9000, 16 000 and 33,000 ×. Negatives were obtained on Kodak Fine Grain Release Positive Film Type 5302 and were enlarged photographically as desired. For the present investigation approximately 750 recordings were studied.

RESULTS

Observations on Negatively Stained Material

Leptospires fixed with osmium tetroxide or glutaraldehyde showed a regular wavy outline of the protoplasmic body. A few blebs were noted along the cells. The wave length generally was about 0.5–0.6 μm. Cells stained after formaldehyde fixation or without fixation showed many blebs. Usually these cells possessed a less regular wavelength and an amplitude smaller than that of cells fixed with OsO_4 or glutaraldehyde. The same result was obtained irrespective of whether the fixatives were diluted in PBS or SMC.

All illustrations show *Leptospira* strain Pomona. Unless otherwise stated, the bar on each micrograph represents 100 nm.

Fig. 1. Osmium fixed organism surrounded by three distinct layers (1, 2, 3). Negative staining with 2 per cent sodium silicotungstate. 115 000 ×.

Fig. 2. Glutaraldehyde fixed organism showing three distinct layers (1, 2, 3) at the tip and only two along the cell body. I denotes the insertion region of the axial filament (F). Negative staining with 1 per cent uranyl formate. 90 000 ×.

Fig. 3. Formaldehyde fixed organism surrounded by two layers (1, 2). The insertion (I) of the axial filament (F) is clearly seen. Some fine transverse striations can be seen on the part of the cell body between the two arrows. The striations are best seen if the page is held obliquely and the picture is viewed in the direction of the arrows. Negative staining with 1 per cent phosphotungstic acid. 115 000 ×.

Fig. 4. Fine striations are visible across the cell (arrow) just above the insertion (I) of the axial filament (F). Note the three layers (1, 2, 3) at the tip of the organism. Glutaraldehyde fixation. Negative staining with 1 per cent ammonium molybdate. 160 000 ×.

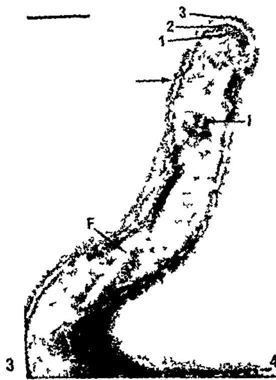
* The purified bacteriolytic enzyme of *Myxobacter* AL 1 protease I was kindly provided by Professor *R. S. Wolfe*, Department of Microbiology, University of Illinois, Urbana, Ill., USA.

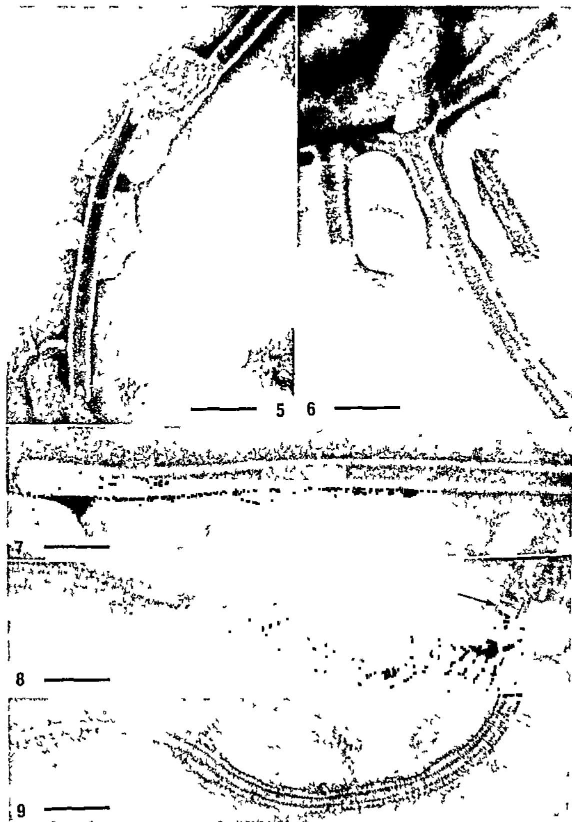
§ YAP medium was made of yeast extract, sodium acetate and peptone 0.3, 0.05 and 0.3 per cent (w/v) respectively, of the Difco products.


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Figs 10 and 11 show cells negatively stained with 1 per cent ammonium molybdate
Fig 10 Two axial filaments inserted at the same end of a leptospira Glutaraldehyde fixation 160 000 ×
Fig 11 A dividing leptospira The two daughter cells are separated by two individual cytoplasmic mem branes (arrows) but still connected by a common cell envelope Note the insertions (I) of the two new axial filaments Formaldehyde fixation 115 000 ×

Figs 5 to 9 all illustrate preparations which are negatively stained with 1 per cent ammonium molybdate

Fig 5 Striated tubule from a preparation treated with Teepol for 2 minutes The tip of a leptospira is seen in the lower left corner 160 000 ×

Figs 6 7 Striated tubules from cells treated in suspension with *Myxobacter* AL 1 protease 1 Parts of two leptospires are seen in the upper left corner of Fig 6 The tubule in Fig 7 is only partly filled with staining material 160 000 ×

Fig 8 Thin striated tubule part of which is in a state of uncoiling (arrow) From a preparation treated with TRIS EDTA buffer only 160 000 ×

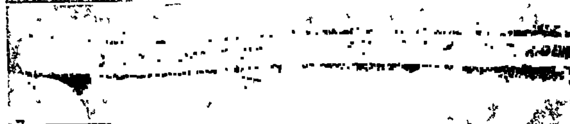
Fig 9 An axial filament parly enveloped by a striated tubule From a preparation of cells heated to 60° C for 1 hour 160 000 ×

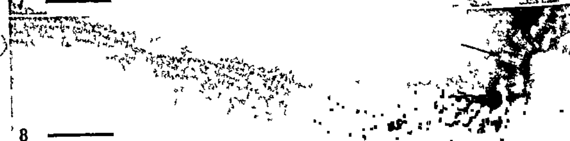
Cells fixed with 0 5 or 0 025 per cent osmium tetroxide clearly showed three distinct layers surrounding the cells (Fig 1) Cells fixed in glutaraldehyde showed evidence of three enveloping layers only at the tips of the cells but two layers along the rest of the cell length (Figs 2 and 4) Cells fixed in form aldehyde, whether 4 per cent or 0 2 per cent, showed only two layers along the entire circumference of the cell (Fig 3)

On close examination of micrographs of some cells a fine transversal striation with a periodicity of approximately 4 5 nm was observed in parts of the cell envelope (Figs 3 and 4) Transversely striated tubular structures with a similar periodicity were found occasionally in preparations of unfixed cells, but were seen more regularly in preparations


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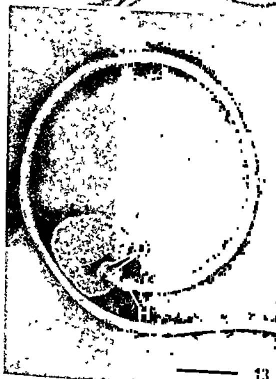

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more well defined electron dense intermediate layer in close association with the outer aspect of the plasma membrane was seen (Fig 16) This intermediate layer was 2–2 5 nm wide

The cell envelope was seen as three electron dense layers with two electron lucent layers in between (Figs 16 and 18) The total width of this five layered cell envelope was 10–11 nm On some obliquely sectioned cells striations with a periodicity of about 4 5 nm were observed in the outer dense layer of the cell envelope (Fig 17) On some transversely sectioned cells this outer dark layer appeared to be punctuated with the same periodicity (Fig 17)

An axial filament was observed in many transversely sectioned cells (Fig 16) It was always found in the interspace between the five layered cell envelope and the intermediate layer situated on the outer leaflet of the cytoplasmic membrane Some micrographs gave the impression of the axial filament being composed of several subfibres, but the actual number of these subfibres could not be ascertained The diameter of the axial filaments in sectioned cells was about 19 nm Convincing evidence of a sheath on the axial filament was not found in sectioned material

Ribosomes were found to be evenly distributed in the cytoplasm of the organisms (Figs 17 and 18) The nuclear regions were distinguishable as less dense regions which contained delicate strands of DNA material (Fig 16) Quite often mesosomes of the whorled type were present (Fig 18)

The results presented above were derived primarily from the study of material fixed with osmium tetroxide alone or after prefixation with glutaraldehyde Cells prefixed with formaldehyde showed a low overall contrast, even after counterstaining of the sections, and the two outermost dark layers of the cell envelope were barely visible (Fig 19)

Figs 16 to 19 all illustrate sections of cells prepared and counterstained as stated in Methods.

Fig 16 The axial filaments (F) are situated between the five layered outer envelope (E) and the intermediate layer (IL) in close apposition to the cytoplasmic membrane (CM) N denotes nuclear region Osmium prefixation 175 000 ×

Fig 17 The obliquely sectioned parts of leptospires show fine striations (S) in the outer layer of the cell envelope In the more transversely sectioned part of the cell this same layer appears punctuated (arrow) The cell envelope (E) and the cytoplasmic membrane (CM) can be seen and ribosomes (R) are present in the cytoplasm Glutaraldehyde prefixation 175 000 ×

Fig 18 A mesosome (M) of the whorled type is present The cytoplasmic membrane appears asymmetric (CM) Ribosomes (R) are evenly distributed in the cytoplasm of the cells Osmium prefixation 160 000 ×

Fig 19 The two outer electron-dense layers of the cell envelope are barely visible in cells prefixed with formaldehyde The cytoplasmic membrane appears asymmetric (arrows) 160 000 ×

DISCUSSION

Leptospires fixed with osmium tetroxide or glutaraldehyde prior to negative staining are apparently better preserved than cells fixed with formaldehyde Even when the former fixatives are used in very low concentrations, the leptospires show regular helices and none or only a few blebs In contrast to these results cells fixed with formaldehyde generally show irregular helices often with nearly straight parts, and on such cells many blebs are visible on the cell surface

In negatively stained leptospires fixed with osmium tetroxide, three layers enveloping the cytoplasmic body of the cell are distinguishable along the entire circumference of the cell On glutaraldehyde fixed cells three layers can be depicted only at the tips of the cells, while two layers were observed on the remainder of the cell body On formaldehyde-fixed cells only two layers seem to be present along the complete cell circumference These discrepant results might be explained by the loss of one layer, or a fusion of two of the layers after aldehyde fixation However, in sectioned cells a five layered cell envelope is always present independent of the prefixative used, this finding indicates that no layer is

lost during fixation with aldehydes as compared to fixation with osmium tetroxide Apparently, a very thorough fixation is required to protect the structures of the cell surface against the surface tension forces during drying for negative staining and this seems to be best effected by osmium tetroxide

Our observation of a five layered cell envelope on *Leptospira* is in agreement with the results of *Pillot* & *Ryter* (20), *Anderson* & *Johnson* (1) and *Auran et al* (2), but in contradiction to those of *Nauman et al* (18) and *Ritchie* & *Ellinghausen* (22), according to whom the cell envelope is triple layered

In some sections, transverse as well as oblique a striated substructure can be observed in the outer layer of the cell envelope A periodicity of 4–4 5 nm was measured, and this is the same as that of the striations seen on a few negatively stained cells The striated tubular structures occasionally observed in between and adjacent to negatively stained unfixed cells show the same periodicity, and so do the tubules very often seen in preparations treated with Teepol sodium deoxy cholate or *Myxobacter* AL 1 protease 1 These observations lead us to the assumption that the striated tubules are in fact pieces of the outermost layer of the cell envelope which are broken off or rolled off from damaged leptospires This assumption is in accordance with the proposal of *Anderson* & *Johnson* (1) that striated tubular structures are either produced by antibody action or by longitudinal breakage of the outer layer of the cell envelope

On the basis of their findings of axial filaments surrounded by striated tubules *Holt* & *Canale Parola* (12) and *Nauman et al* (18) proposed that the axial filament consists of a sheathed core enveloped by a striated tubule However the width of this axial filament striated tubule complex is not in agreement with the width of the axial filaments as measured on the illustrat ons of well preserved negatively stained v le cells or with the diameter of the axial fila n s in the sectioned cells illustrated in the pers mentioned above It cannot be exclu that by chance some axial filaments are enclosed in fragments of the outermost layer of the cell envelope when this layer breaks during damage to the cell This opinion has also been expressed by *Chang* & *Faine* (9)

Our observations on the dimensions and the substructures of the core, the sheath (thickness 3 nm as calculated from the difference in overall width of sheathed and unsheathed filaments), and the hook are in accordance with earlier observations on axial filaments of *Leptospira* (18, 20) and on flagella of *Treponema* spp (5, 14, 15, 20) In addition the results are in accordance with observations on corresponding structures on flagella isolated from gram negative bacteria (see *DePamphilis* & *Adler* (10) for references)

The finding that the distal 1/3 1/8 of the axial filament is left unsheathed is in agreement with the observations of *Babudieri* & *Bocciarelli* (4)

The dimensions and the mutual positions of the basal discs on the axial filaments of the parasitic leptospires of the present study are the same as those found by *Nauman et al* (18) on axial filaments of the saprophytic *Leptospira* strain B16 The size and the positions of the basal discs are also very similar to those of the basal discs of flagella isolated from gram negative bacteria (10) It thus seems reasonable to consider these morphological elements as being structurally analogous and consequently to consider the axial filaments of *Leptospira* as flagella The main difference between the basal body of leptospiral flagella and the basal body of flagella from gram negative bacteria (10) is that on the leptospiral flagellum the L ring is barely visible and the diameters of the S and M rings are larger than those of the S and M rings on flagella from gram negative bacteria (see *DePamphilis* & *Adler* (10) for explanation of nomenclature)

Chemical analysis of isolated axial filaments of *Leptospira* strain B16 (18) showed their composition to be similar to though not identical with that of isolated flagella of *Proteus vulgaris* Differences in chemical

composition may explain why isolated leptospiral axial filaments tend to coil up in a spiral, which is not the case either for flagella isolated from bacteria or from *Treponema* spp (10, 13, 14) At present we have no evidence to prove or disprove whether this coiling capacity of the axial filaments is linked to either the core or the sheath or to both of these structural elements

The helical shape of the leptospiral cell is preserved after treatment, *e g* with Teepol, which ruptures the cell envelope and liberates the axial filaments Such treatment leaves the intermediate layer adhering to the outer leaflet of the cytoplasmic membrane intact This layer is probably the mucopeptide layer which constitutes the rigid part of the cell-wall complex The exterior part of this complex, the five layered envelope, seems to consist of an ordinary triple-layered membrane with an additional structured layer exterior to it In this way the architecture of the leptospiral envelope is quite similar to that of several *Spirillum* spp (7, 8) in which a regular surface layer of a proteinaceous nature is located on the exterior side of a triple layered lipoprotein lipopolysaccharide membrane and separated from it by an electron-translucent layer of unknown composition

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The authors wish to thank Mrs *J Berg*, Mrs *H Rain* and Mr *F Laursen* for their excellent assistance in sectioning and electron microscopy Miss *A G Overgaard* and Mr *F Laursen* are thanked for expert photographic work

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ULTRASTRUCTURE OF A MYCOPLASMA-LIKE AVIAN CONJUNCTIVITIS AGENT

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Based on electron microscopical studies of an avian conjunctivitis agent propagated in cell cultures and fixed in situ, the agent has been found to be a mycoplasma like organism, morphologically and ultrastructurally resembling *Mycoplasma gallisepticum* Previously described caracteristica of the agent do not exclude that the agent taxonomically is placed in the mycoplasma group, but additional investigations are needed for a correct placing

In previous publications (*Velling* 1957, 1962, 1970) an eye disorder was described which mainly occurred among broilers in Denmark during the years 1953 to 1965 The disease could be reproduced by an isolated agent, but attempts to identify the agent were unsuccessful The agent could be demonstrated in cell cultures and chick embryos, but several factors, including the size of the agent and particularly its sensitivity to antibiotics, excluded the possibility of a virus Its possible relationship to the Bedsonia group was also studied, but in spite of the fact that it had certain properties in common with this group, it deviated from it in other respects Thus, no cross-reactions between the agent and known Bedsonians could be demonstrated in complement fixation tests

Amongst known infectious agents the mycoplasma group still remained a possibility, but attempts to grow the conjunctivitis agent in conventional mycoplasma media were unsuccessful Propagation only took place in the yolk sac of chick embryos and in chick embryo kidney tissue cultures It seemed possible that electron microscopy might provide information of value for the correct taxonomic placing of the agent Therefore a study of chick embryo kidney cell cultures infected with the agent was carried out by means of electron microscopy and the following report contains the results obtained

MATERIALS AND METHODS

Agent Strain No 528 used in the study was originally isolated in the yolk sac from a typical field outbreak of infectious conjunctivitis in a chicken and re isolated from an experimentally infected chicken showing typical clinical symptoms The strain was transferred to chick embryo kidney tissue cultures and was used in the 4th an 7th cell culture passage Embryonated eggs as well as chick embryo kidney tissue cultures used for primary isolation and propagation of the conjunctivitis agent were derived from mycoplasma free and otherwise healthy flocks (*Velling* 1970)

Tissue cultures The chick embryo kidney tissue cultures were prepared as previously described

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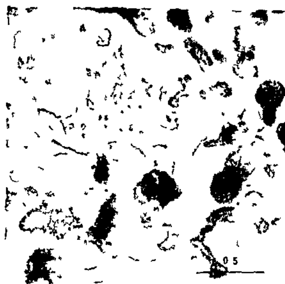


Fig 1 Cluster of organisms associated with cellular debris Length unit used on all figures is μm

(*Velling* 1970) The cells were grown in Leighton tubes and the inoculated cultures as well as the non inoculated controls were fixed 72 hours after inoculation

Electron microscopy The cells were fixed in situ in 2 5 per cent glutaraldehyde in 0 1 M phosphate buffer (pH 7 3) for two hours at room temperature and left overnight in 1 per cent osmium tetraoxide in the same buffer The cultures were dehydrated in acetone and embedded in Vestopal W (Serva) according to *Ryter & Kellenberger* (1958)

After about two hours in the final embedding mixture, the coverslips were transferred to aluminum trays which were easily removed after polymerization The coverslips were then separated from the vestopal blocs under binoculars, leaving the cells in the vestopal blocs Sections were cut on an LKB ultratome I and contrasted with 1 per cent uranylacetate and lead citrate according to *Reynolds* (1963) They were examined in a JEM 100 B electron microscope

RESULTS

While controls remained uninfected, the agent was found in all preparations of infected cultures attached to the surface of the culture cells, especially to older, decaying cells and clusters of cellular debris The attachment was often found to be on uneven surfaces or in pockets between the cells, whereas flat culture cells with a smooth and even surface seemed less attractive to the agent

The agent had polymorphic shapes from regular pear and spindle shape to undulated bowl-shape—often with irregular protrusions (Fig 1) At one end, the front end, the agent had a rigid bud, in the mycoplasma nomenclature called a bleb, to which the rest of the cell was attached like a plastic bag usually with a constriction between the rigid bud and the rest of the cell Because of its plastic property, the agent was able to adapt itself to the shape of the surface to which it adhered In its most regular shape (Fig 2), the agent was 0 6–0 7 μm long and approximately 0 25 μm broad

The Membrane

The agent was covered all over by a trilaminar plasma membrane, whose outer layer seemed to be thicker than the inner layer, no doubt due to a coating on the outer surface

The inner layer of the plasma membrane was difficult to distinguish from the very osmiophilic and compact contents, and it was most clearly visible in—presumably—old organisms, whose contents seemed to be denaturated and aggregated into a nucleus like dense mass in the centre of the spherical body The plasma membrane without its coating was approximately 10 nm thick

The Bleb

The diameter of the bud shaped bleb was approximately 135 nm Close behind the somewhat flattened front end of the bleb an 8 nm thick bowl shaped plate was situated To this was anchored by a number of fine connections, a lens shaped body with dense osmiophilic walls surrounding a less osmiophilic lens shaped space in the centre of the bleb (Fig 3)

The Infrableb Region

The part of the body adjacent to the bleb was, in its most regular form, a truncated cone pointing towards the bleb The plasma in this region was almost homogeneous at

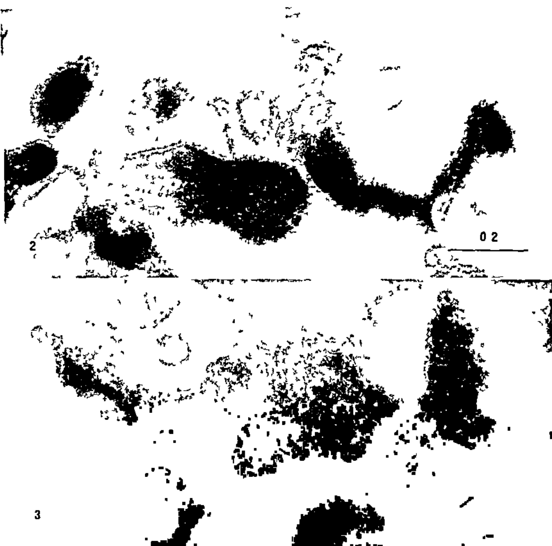


Fig 2 Part of Fig 1 showing *Mycoplasma gallisepticum*—resembling organisms one of which is of a most regular shape Bleb infrableb region and vacuole are recognized in the organism on the right

Fig 3 Part of Fig 1 Organisms with ribosomes scattered at random in the outer part of the rear end

lower magnification and finely dotted, containing faint tubules at higher magnification, and it appeared to be less osmiophilic than the cell membrane This part of the cell is identical to the infrableb region in the mycoplasma nomenclature

Ribosomes and DNA

The rear part of the body was strongly osmiophilic The outer region near the membrane contained a large number of ribosomes surrounding a core, most of which contained long filiformed molecule complexes proceeding from the infrableb region and protruding backwards lengthways in the cell

Most frequently the ribosomes were scattered at random in the region containing them, however, in a few cases they were arranged in closely spaced cylindrical or helical formations (Fig 4 and 5) The num-

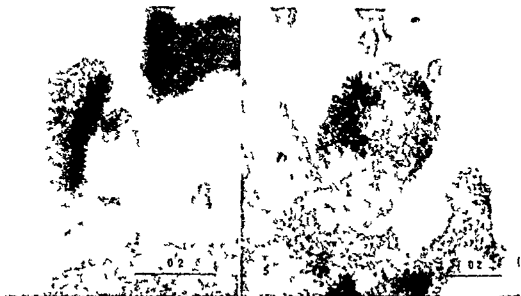


Fig 4 Polyr bosome l ke structures long tud nally cut Note also the filamentous central contens in the organ sm in the corner

Fig 5 Polyribosome l ke str ictures l or zontally cut

ber of ribosomes per turn of the helix and the total number of turns could not be de termined with sufficient accuracy from the present data

Vacuoles

Quite often the cells contained vacuoles surrounded by a trilaminar membrane of the same asymmetric appearance as the plasma membrane and apparently formed by an engulfing of the growth media (Fig 6) The vacuoles were formed in the region contain ing ribosomes but were also found on the border of the infrableb region Apparently the vacuoles collapsed subsequent to the ab sorption of their content Some findings seemed to indicate that emptied and col lapsed vacuoles were returned into the media (Fig 7)

As mentioned earlier the agent cells were positioned in contact with the surface of the culture cells Sometimes the bleb could be the only point of contact (Fig 8) but more frequently a zone of the infrableb region accomplished the contact and the bleb was tilted to one side The part of the cell con taining ribosomes was then bent into the medium and it was quite often seen to be engulfing some media The rear end could project out forming a slender process (Fig 9)

The outer layer of the plasma membrane of the agent cell sometimes showed fine per forations and fine diffuse connections or bridges could be observed between the two plasma membranes (Fig 10) Similar changes were not discernible with certainty in the plasma membrane of the culture cell No observations indicated any distinct mode of reproduction

DISCUSSION

Using the embedding technique described one could expect to obtain an impression of the agent as it appears in its normal environ ment except of course that the majority of the non adhering organ sms are most prob ably removed if the medium is changed etc

Some of the free unattached organisms must however still remain arrested in pock ets between the culture cells Also it can be

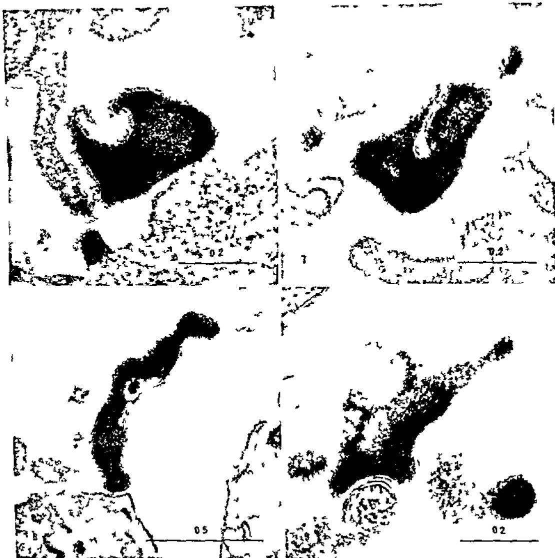


Fig. 6. A commonly found configuration of processes from the rear part of the agent. Ribosomes are situated in the periphery surrounding the horizontally cut interior.

Fig. 7. Collapsed vacuole lying in the ribosome containing part of the organism.

Fig. 8. Organism making contact with cellular surface. Bleb, infrableb region and vacuole are clearly visible.

Fig. 9. Organism of a highly irregular shape in contact with cellular protrusions.

assumed that structurally the agent is rather well preserved when the cells are fixed in situ without any previous separation procedure.

In its most regular shape the agent exhibits great similarity in size and shape to *Mycoplasma gallisepticum* when the latter is fixed in situ with glutaraldehyde as described e.g. by *Maniloff et al.* (1965 a) working with strain A 5969 as well as *Munkres & Wachtel* (1967) also working with strain A 5969. In these investigations the cells were in general more regularly shaped pre-



Fig 10 Organism in contact with cellular protrusion Note the perforated outermembrane of the organism in the contact zone

sumably, because the examined strains were propagated in artificial media The *M gallisepticum* is depicted as a pear- or drop-shaped cell, 0 6–0 7 μm in length, with (1) a bleb at one end (the front end), (2) a homogeneous interregion—the so called infrableb zone, and (3) a bag shaped ribosome containing rear part, 2–2 5 times as long as the bleb and infrableb region

The bleb region is described in detail by *Maniloff et al* (1965 a) and apart from the fact that the body we designate lens shaped is called plate shaped by them, their observations are in good accordance with our findings Also their description of the infrableb region is in close agreement with our observations

As mentioned earlier we find that the adhesion of the agent cell to the surface of the culture cell takes place at the surface of the bleb and infrableb region *Munkres & Wachtel* (1967) showed that these regions contain ATPase and that the infrableb region contains acid phosphatase These observations may explain just why the adhesion takes place at the positions mentioned

The ribosomes of *M gallisepticum* A 5969 are found e g , by *Maniloff et al* (1965 a) to be 14 nm in diameter, and in cells fixed before centrifugation the ribosomes are found distributed at random in the rear part of the cell between the plasma membrane and the centre part The latter, as shown by *Maniloff et al* (1965 a, b), contains fibrillar and granular material identical to DNA This corresponds closely to our observations We also find that the ribosomes in a few cells are arranged in a number of spirals or cylindrical configurations, and similar observations are reported by *Allen et al* (1970) in *M gallisepticum* strain 1056, by *Domermuth et al* (1964) in *M gallisepticum* strain JA and by *Maniloff et al* (1965 b) in *M gallisepticum* strain A 5969, in the majority of logphase cells when centrifugation is carried out before fixation More recently the same structures have also been found in human T mycoplasmas by *Black et al* (1972)

Our data do not indicate whether these spirals, cylindrical formations or polysomes are present in cells that have been injured in one way or another, or whether these configurations are connected with some short term phase in the life cycle of the cell

The plasma membrane of *M gallisepticum* is described by *Domermuth et al* (1964) and by *Maniloff et al* (1965 a, b) as an 11 nm thick unit membrane having outer layers, which each measure 3 nm in thickness, and a middle layer 5 nm thick

The adsorbed material on the outer layer which gives the unit membrane an asymmetric appearance, is also described by *Domermuth et al* (1964) The same asymmetric membrane also lines the inclusions or vacoules, described previously in *M gallisepticum* strain 1056 by *Allen et al* (1970) All their circumstantial evidence is in accordance with our observations

The present ultrastructural investigation indicates that our agent may belong taxonomically to the mycoplasma group—more precisely to a mycoplasma ultrastructurally resembling *M gallisepticum*, which in the tentative morphotype grouping of *Maniloff & Morowitz* (1972) is placed as their V category, caracterized as ' pear shaped cells with

terminal structures, the only known examples are the blebs of the strains of *M gallisepticum*"

Previously described caracteristica of the agent do not exclude the agent from the mycoplasma group but they do not give sufficient evidence for a correct placing Therefore, until more data are collected the agent can only tentatively be identified as a mycoplasma like organism, morphologically and ultrastructurally resembling *M gallisepticum*

The technical assistance of Mrs *I S abo* and Mrs *U Borgesen* is highly appreciated Thanks are also due to *V Middelboe* Lic scient et agro for advice concerning the translation of the manuscript

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RAPID IDENTIFICATION OF *PROTEUS* SPECIES AND *PROVIDENCIA* BY A SIMPLE TWO-STEP PROCEDURE

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One hundred and sixty nine strains of *Proteus* and *Providencia* were studied A simplified identification procedure in two steps was applied In the first step, strains belonging to the *Proteus Providencia* group were identified on the basis of one hour tests for phenylalanine deaminase and β D galactosidase activity In the second step, differentiation between the four *Proteus* species and *Providencia* was based on H_2S production, urease activity fermentation of maltose and ornithine decarboxylase activity (HUMO) Identification based on the HUMO scheme accorded well with the final identification based on 19 characters The HUMO scheme is therefore recommended for use in routine diagnostic work

In routine bacteriology there is a great need for simple identification schemes for the most commonly occurring genera of *Enterobacteriaceae* We have earlier proposed a scheme for the identification of the prompt lactose-fermenting genera (6) based on the four characters H_2S production ornithine decarboxylase activity, mobility and citrate utilization (HOMoC)

Turning to the genera of *Enterobacteriaceae* fermenting lactose slowly or not at all, *Proteus* and *Providencia* probably constitute the most frequently isolated groups (19) Yet little work has been done to select a restricted number of characters which would allow a reliable diagnosis of these species in routine diagnostic work The criteria used have probably varied from one laboratory to another and this may have led to some confusion, especially concerning the proper identification of the various *Proteus* species and the differentiation of *Providencia* from other 'Coliforms

In the present paper experience with a new identification scheme for the genera *Proteus* and *Providencia* is described The identification was carried out in two steps the first step served to separate two genera from other non-lactose fermenters, the second to differentiate among *Proteus* species and *Providencia*

MATERIAL AND METHODS

Strains 226 strains were isolated from routine clinical specimens 210 of them in the period September 1969 to April 1970 In addition 16 reference strains of *Proteus morgani* obtained from NCTC were included

Primary isolation was made on nutrient agar plates containing 1 per cent lactose 0 1 per cent sodium thiosulphate 0 005 per cent crystal violet and 0 008 per cent bromthymol blue In order to obtain pure culture, colonies of non lactose fermenting strains were picked from these plates after overnight incubation

Identification Criteria for identification were based on *Cowan & Steel* (7) and *Edwards & Ewing* (10)

Media and reagents β D galactosidase activity

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TABLE 1 *Biochemical Reactions of 169 Strains of* Proteus *and* Providencia

or rate	*Proteus mirabilis* +	*Proteus mirabilis* —	*Proteus vulgaris* +	*Proteus vulgaris* —	*Proteus morgani* +	*Proteus morgani* —	*Proteus rettgeri* +	*Proteus rettgeri* —	*Providencia* +	*Providencia* —
G	0	64	0	28	0	34	0	23	0	20
	64 (1)	0	28 (4)	0	34 (6)	0	23 (2)	0	20	0
ming	57	7	13	15	0	34	0	23	0	20
ose (gas)	64	0	23 (9)	5	34	0	9 (1)	14	(3)	17
se	64	0	17	11	0	34	1 [2]	20	3	17
ose	0	64	28	0	0	34	0	23	0	20
itol	0	64	0	28	0	34	20	3	2	18
nitol	0	64	0	28	0	34	17	6	0	20
tol	0	64	0	28	0	34	12 [7]	4	14 (1)	6
	0	64	0	28	0	34	0	23	0	20
A	64	0	0	28	34	0	0	23	0	20
	62	2	13 (1)	15	0	34	0	23	0	20
lity	64	0	23	5	32	2	13	10	10	10
ate	50 (20)	14	5 (3)	23	2 (1)	32	7 (1)	16	20 (1)	0
N	64	0	28	0	31	3	20	3	20	0
ase	64	0	28	0	34	0	23	0	0	20
tin	58 [2]	4	14 [13]	1	0	34	0	23	0	20
ol	0	64	15	13	34	0	23	0	20	0
ate	64	0	28	0	33	1	23	0	20	0

Symbols + = positive reaction — = negative reaction, () = weakly positive reaction, [] = positive reaction after 48 hours, ONPG = β D galactosidase activity, PAD = phenylalanine deaminase activity, LDA = lysine decarboxylase activity, ODA = ornithine decarboxylase activity

(ONPG) and phenylalanine deaminase activity (PAD) were detected in the same tube A paper strip impregnated with 1 per cent L-phenylalanine solution (12) was inserted into a heavy suspension of bacteria in 0 5 ml ONPG broth (15) The tube was incubated for 1 hour at 37° C before the ONPG test was read Immediately afterwards, 1 drop of 10 per cent $FeCl_3$ was applied to the paper strip Green colour on the strip indicated deamination of phenylalanine For the other reactions the following media were used Fermentation of glucose, xylose, maltose, adonitol, mannitol, and inositol (meat extract broth containing 1 per cent carbohydrate and bromthymolblue indicator), lysine (LDA) and ornithine decarboxylase activity (ODA) (Decarboxylase Medium (Difco) with 0 3 per cent agar and 1 per cent lysine and ornithine respectively (14)), a combined medium for detection of H_2S production and motility (5), citrate utilization (Simmons medium (7)), KCN test (17), urease activity (4), gelatin hydrolysis (Difco Nutrient Gelatin 128/1), indol production (10), nitrate reduction (8) with 0 1 per cent nitrate All these tests were read after overnight incubation at 37° C The only exception was the test for gelatin hydrolysis which routinely was read both after 24 and 48 hours If a test was difficult to read it was repeated and read both after 24 and 48 hours

RESULTS

Initially all strains were tested in the PAD and the ONPG reaction One hundred and sixty-nine strains were able to deaminate phenylalanine but did not show β-D-galactosidase activity According to *Edwards* & *Ewing* (10) these strains belonged to the *Proteus-Providencia* group Among the remaining 73 strains 61 were negative in the PAD test and positive in the ONPG test On the basis of the reactions in the HOMoC scheme (6) 48 were identified as *Escherichia*, 11 as *Enterobacter* and 2 as *Klebsiella* Twelve strains were negative in both reactions According to directions given by *Cowan* & *Steel* (7) these were identified as 9 *Pseudomonas aeruginosa*, 2 *Alcaligenes faecalis* and 1 *Acinetobacter anitratus*

The biochemical reactions of the 169 strains belonging to the *Proteus-Providencia* group are given in Table 1 Each strain was identified on the basis of all reactions The

TABLE 2 *Reaction Pattern of 169 Strains of Proteus and Providencia in the HUMO Scheme*

H	U	M	O	Number of strains
+	+	—	+	62 *Proteus mirabilis*
—	+	—	+	34 *Proteus morgani*, 2 *Proteus mirabili*
+	+	+	—	13 *Proteus vulgaris*
—	+	+	—	15 *Proteus vulgaris*
—	+	—	—	23 *Proteus rettgeri*
—	—	—	—	20 *Providencia*

Symbols + = positive reaction, — = negative reaction, H = hydrogen sulphide production, U = urease activity, M = fermentation of maltose, O = ornithine decarboxylase activity

strains showing an atypical pattern are discussed below

Proteus mirabilis Fourteen strains (22 per cent) could not utilize citrate as the sole source of carbon, and 20 strains showed only faint growth Seven strains (11 per cent) did not swarm on the medium used Six strains (9 per cent) did not liquefy gelatin after 24 hours, 2 strains becoming positive after 48 hours Two strains did not produce H_2S Only 4 strains were aberrant in 2 reactions

Proteus vulgaris Swarming could not be detected in 15 strains (54 per cent) and 5 of these were also non motile Fifteen strains (54 per cent) were H_2S negative Thirteen strains (46 per cent) did not produce indol Five strains (8 per cent) did not produce gas from glucose One strain did not liquefy gelatin and 13 did not become positive until after 48 hours Two strains were aberrant in 5 of these reactions 3 strains in 4, 4 strains in 3 and 7 strains in 2 reactions

Proteus morgani Two strains (6 per cent) were non motile 1 strain did not reduce nitrate to nitrite but showed no other aberrations

Proteus rettgeri Sixteen strains (70 per cent) did not grow on the citrate medium Motility was not detected in 10 strains (43 per cent) Six strains (26 per cent) did not ferment mannitol Four strains (17 per cent) were inositol negative and 7 did not become positive until after 48 hours Three strains (13 per cent) failed to ferment adonitol Two strains showed aberrations in all these 5 reactions, 1 strain in 4, 1 strain in 3 and 8 strains in 2 reactions Three strains did not ferment mannitol, inositol or adonitol

Providencia Apart from the fact that the percentage of motile strains (50 per cent) was somewhat lower than expected, only minor aberrations in the reaction pattern were found

After identifying the 169 strains on the basis of the data given in Table 1, we selected four characters which seemed to provide an equally accurate basis for identification When the group diagnosis *"Proteus Providencia"* had been made from the presence of phenylalanine deaminase activity and the absence of β D galactosidase activity, we found that the combination of urease activity, H_2S production, ornithine decarboxylase activity and fermentation of maltose constituted an effective, simplified identification scheme for routine use Identification of the 169 strains according to these four criteria is given in Table 2 As shown, the differentiation was clear-cut expect for two strains of *P mirabilis* which could not be distinguished from *P morgani*

DISCUSSION

We consider that the most reasonable way of reducing the workload of the medical bacteriological laboratory without impairing the diagnostic standard is to apply simplified diagnostic schemes based on a restricted number of characters It seems preferable to establish such schemes for each of the most commonly occurring groups of species

rather than to use a standard identification scheme for the differentiation of a large number of species

In the period September 1969 to April 1970, when most of our material was collected, the *Proteus Providencia* group accounted for 65 per cent of the non lactose fermenting isolates, and among all isolates those belonging to this group were second in frequency only to *Escherichia*

The present study has been carried out to provide experience with a simplified test battery for the diagnosis of *Proteus* and *Providencia* In the selection of tests we have considered only those that can be read after 24 hours, that are easy to read, and give clear-cut criteria for the bacteriological diagnosis An attempt has been made to ascertain the minimum number of tests necessary to obtain an accurate identification of the isolates

Since non lactose-fermenting Gram negative rods include a wide variety of species we have found it convenient to carry out the identification in two steps In the first step it is determined whether or not the isolate belongs to the *Proteus Providencia* group, based on the ONPG and the PAD test These tests were selected because they yielded reliable results after short term incubation and because nearly 100 per cent of *Proteus* and *Providencia* strains are PAD positive (9, 13, 19) and ONPG negative (19) and to our knowledge, the only other species able to deaminate phenylalanine is *Moraxella phenylpyrouvica* (3)

Final identification of the strains was based on 19 characters (cfr Table 1) The selected four characters used in step two yielded results that were identical with the final identification with one exception, they failed to differentiate between *P morgani* and non swarming, H_2S negative strains of *P mirabilis* This may be achieved by including a test for indol production

Considering the limited number of strains included in our study, the reaction pattern of each species is with one exception in reasonable agreement with the data provided by *Edwards & Ewing* (10) The only important discrepancy is the higher frequency of indol- and H_2S negative *P vulgaris* strains in our material, 11 strains (39 per cent) were negative in both reactions This might have been due to lack of sensitivity in our tests, but the medium used for indol production was the one recommended by *Edwards & Ewing* (10), and all the *P vulgaris* strains being H_2S negative were retested in triple sugar iron agar (10) and read both after 24 and 48 hours An accumulation of one particular strain of *P vulgaris* among our isolates might also have been a cause of the discrepancy However, the 17 strains found to be negative in one or both reactions were isolated from specimens sent to the laboratory from eight different hospitals and general practitioners in Western Norway Only two strains with identical reaction pattern came from the same clinical department Finally, our identification of these strains as *P vulgaris* might have been wrong, but their biochemical reaction pattern seemed strongly to contradict the possibility that they belonged to another *Proteus* species

We therefore assume that our final identification of the strains in all essentials is correct, and there is no evidence indicating that nosocomial spread of one particular strain has influenced our results As regards the indol reaction, our findings are in accord with the observations by *Matsen et al* (16) who found tests for indol production less reliable than tests for ODA for a distinction between *P mirabilis* and *P vulgaris*

Different *Proteus* species vary considerably in their susceptibility to antibiotics, but as a rule strains of *P mirabilis* are inhibited by lower concentrations of cephalosporins and penicillins than strains of the other species (1, 2, 18) Since the indol reaction has often been the only one referred to when the antibiotic sensitivity pattern of *Proteus* is discussed (11, 20) it will be of interest to see whether the general sensitivity pattern of the *Proteus* species is maintained when our diagnostic criteria are used

In this study, identification of *Proteus* and *Providencia* strains has been successfully

made on the basis of the four characters present in the "HUMO" scheme This scheme is only meant to provide a basis for simple and reliable identification in routine diagnostic work For epidemiological purposes a further characterization of the strains will be needed, including additional biochemical characteristics, antibiogram, and other biological markers (1)

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OCCURRENCE OF OCHRATOXIN A AND CITRININ IN CEREALS ASSOCIATED WITH MYCOTOXIC PORCINE NEPHROPATHY

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A survey of the nephrotoxic mycotoxins ochratoxin and citrinin in cereals used as feed for bacon pigs was conducted, employing thin layer chromatography techniques, in districts of Denmark where the incidence rate of mycotoxic porcine nephropathy was high A total of 58 per cent of the samples contained ochratoxin A and 9 per cent contained citrinin, always together with ochratoxin A The detection of the two mycotoxins was confirmed by deriva tive formation and, for selected samples, by nuclear magnetic resonance spectroscopy (NMR) The maximal concentration of ochratoxin A and citrinin was 27 5 ppm and 2 ppm, respectively About half of the contaminated samples contained more than 200 ppb, which is the level at which development of porcine nephropathy is possible This is the first report which elucidates the association of ochratoxin A and citrinin and a naturally occurring disease in domestic animals

Aetiological studies of mycotoxic porcine nephropathy resultated in the isolation of a nephrotoxic strain of *Penicillium viridicatum* West. (Strain no 67 B) from a nephrotoxic batch of barley (*Krogh & Hasselager* 1968) Chemical investigations of nephrotoxic fungal metabolites, using rats as test animals, revealed the strain of *P viridicatum* to be a producer of citrinin (Fig 1) and oxalic acid (*Krogh et al* 1970) By feeding crystalline citrinin to swine a nephropathy similar to the naturally occurring porcine nephropathy was developed, whereas it proved impossible to reproduce nephropathy by feeding oxallate to swine (*Friis et al* 1969)

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The observation that *P viridicatum* includes producers of citrinin has subsequently been confirmed (*Scott et al* 1970, *Scott et al* 1972) On several occasions, the same species has been found to be a producer of ochratoxin A (Fig 2) (*Walbeek et al* 1969, *Scott et al* 1970, *Ciegler et al* 1972, *Scott et al* 1972)

The aforementioned strain of *P viridicatum* (no 67 B) is also able to produce ochratoxin A (*Krogh & Hald,* unpublished results) As ochratoxin A exhibits the toxicity primarily through a damage to the proximal renal tubuli (*Purchase & Theron* 1968) it was considered reasonable to include ochratoxin A as well as citrinin as potential agents of mycotoxic porcine nephropathy This assumption was strengthed by the detection of ochratoxin A and citrinin as natural contaminants of the batch of barley from which

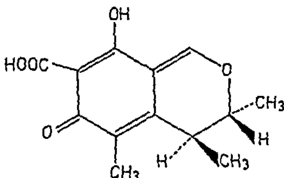


Fig 1 Citrinin

strain 67 B of *P viridicatum* was isolated The same batch of barley had 7 years earlier been used as feed during a successful reproduction of mycotoxic nephropathy in swine (*Nielsen & Hasselager* 1965)

In order to elucidate the possible involvement of citrinin and ochratoxin A in the aetiology of mycotoxic porcine nephropathy it was decided to conduct a screening for these two mycotoxins in cereals associated with field outbreaks of this particular kidney disease

MATERIALS AND METHODS

Collection of Samples

Pig farms with recent outbreaks of mycotoxic nephropathy were traced through the Central Registration of Mycotoxic Porcine Nephropathy (MPN) This institution was established through the Danish Veterinary Service in 1969 and collects monthly reports of observed cases of MPN from the meat inspectors of all Danish slaughterhouses. Samples (0 5 kg) of cereals used as pig feed were collected

Mycotoxin Analysis

Ochratoxin analysis was carried out according to the method of *Steyn & van der Merwe* (1966), involving extraction with methanol-chloroform in Soxhlet apparatus, purification by extraction with aqueous bicarbonate, and thin layer chromatography (TLC), by the use of qualitative and quantitative standards of ochratoxin A and B The sensitivity limits of the assay carried out were 10–20 ppb ochratoxin A

Citrinin analysis was performed according to the method of *Hald & Krogh* (1973), involving extraction with chloroform after acidification, concentration of the extract, and subsequent thin layer chromatography, by the use of qualitative and quantitative standards of citrinin The sensitivity limits of the assay carried out were about 200 ppb

The presence of mycotoxins from samples that appeared to be positive by TLC was confirmed by derivative formation, and for selected samples by NMR spectral analysis

Derivative Formation

Methyl esters of ochratoxin A was prepared according to the method of *Nesheim* (1969), from all samples containing ochratoxin A

Formation of the acetate of citrinin was carried out according to the method of *Hald & Krogh* (1973), from all samples containing citrinin

NMR Spectral Analysis

This analysis was carried out on a sample of TLC purified citrinin from one citrinin containing sample (no 17) and on a sample of TLC purified ochratoxin A from one ochratoxin A containing sample (no 5) The ^{1}H NMR spectra were recorded at 100 MHz on a Varian HA 100 D instrument in connection with a Varian 620/i computer (spectro system 100) The spectrometer was locked on tetramethylsilan (TMS) and the

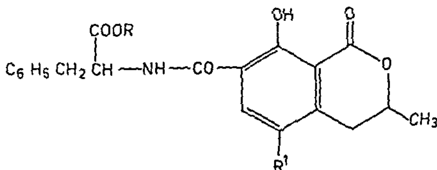


Fig 2 Ochratoxin A R = H, R^1 = Cl
Ochratoxin B R = H R^1 = H
Ochratoxin C R = CH_2CH_3 R^1 = Cl

TABLE 1 *Incidence Rate of Mycotoxic Porcine Nephropathy (Cases pr 100 000 Slaughtered Pigs)*

	District I (Kalundborg)	District II (Svendborg)	District III (Aars)	Denmark mean value for 1971
April 1971	479	255	346	
May 1971	1068	229	986	67
June 1971	695	144	604	

spectra measured in deuterochloroform (99 8 atom per cent D) relative to TMS at 31° C Because of the small amount of sample it was necessary to use the time average of 150 to 1000 scans, in order to get a reasonable signal to noise ratio

RESULTS

The 3 districts were selected because of the high incidence rate of MPN there (Table 1), and represent 3 main areas of Danish agriculture The collection of samples of cereals was carried out in May-June 1971, except that the collection of samples in district III was postponed until September 1971 The control samples, all barley were collected from batches considered to be of high quality All samples were drawn at random, and do not necessarily represent completely the batches from which they were taken

Ochratoxin

Ochratoxin A was detected in 22 samples (Table 5), whereas ochratoxin B, C and esters were not detected The incidence of ochratoxin A in cereal samples was in the range 50–65 per cent in the districts in which the incidence rate of mycotoxic porcine nephropathy was high, compared to 6 per cent ochratoxin contaminated samples of control barley (Table 2) As expected, the pronounced high incidence of ochratoxin A in samples from all-districts (I+II+III) differs statistically significant from the control samples ($P < 0\,001$, see Table 3) Also calculations of the 3 districts individually show, as expected, the non-random distribution of high incidence of ochratoxin A in cereal samples compared to control barley, resulting in $P < 0\,001$ for district 1 and district 2, and $P < 0\,005$ for district 3

The distribution of ochratoxin A, according to concentration, is indicated in Table 4 As levels about 200 ppb seem to be the lowest level causing nephropathy in pigs during 3–4 months of exposure (*Krogh et al* 1973, in preparation), it is essential to note that 50–60 per cent of the contaminated samples from the districts with high MPN incidence rates contain more than 200 ppb, whereas the highest concentration of ochratoxin A in the control was 189 ppb (Table 5)

The actually detected levels in the samples are shown in Table 5 which indicates that the highest concentration detected was 27520 ppb

TABLE 2 *Incidence of Contaminated Samples of Cereals*

	Number of samples examinated	Percentage of samples containing	
		Ochratoxin A	Citrinin
District I	19	65	11
District II	10	50	10
District III	4	50	0
All districts (I + II + III)	33	58	9
Control samples	50	6	N E *

* N E = Not examined

44*

TABLE 3 *Statistical Comparison of Cereal Samples from all Districts (I+II+III) to Control Samples by Use of "2×2 table"*

	All districts (I+II+III)	Control samples	Total
Number of ochratoxin contaminated samples	19	3	22
Number of non-contaminated samples	14	47	61
Total number	33	50	83

$x^2 = 27.1 \sim P < 0.001$

TABLE 4 *Distribution of Ochratoxin A*

Level of ochratoxin A (ppb)	Percentage of ochratoxin contaminated samples				
	District I	District II	District III	All districts (I+II+III)	Control
< 200	58	40	50	53	100
200–1000	17	0	50	16	0
> 1000	25	60	0	31	0

Citrinin

Citrinin was detected in 3 samples (Table 5), resulting in an all-district incidence of 9 per cent (Table 2) The level of citrinin was in the range 160–2000 ppb (Table 5), which is much lower than that required for the development of porcine nephropathy by citrinin alone (*Friis et al* 1969)

Confirmation

The presence of ochratoxin A and citrinin was confirmed for all positive samples by derivative formation

The ^{1}H NMR spectrum of ochratoxin A, which was dissolved both in deuterochloroform and in dideuterodichlormethane (99 atom per cent D) in order to observe the aromatic protons in the phenylalanine residue showed a content of ochratoxin A, as seen by comparison with an autentical sample and in agreement with *van der Merwe et al* (1965)

Because of unknown impurities with strong lines in the low field region of the spectrum it was impossible to measure lines with δ smaller than 3.2 ppm But the existence of those protons was found by their spin couplings to the other protons

In the ^{1}H NMR spectrum of citrinin, all chemical shifts and coupling constants of autentical citrinin were reproduced (*Mathieson & Whalley* 1964) The up-field half of the doublet assigned to the methyl residues at the C-4 atom was not measured because it coincides with a relative large line coming from an unknown impurity, at δ = 1.28 ppm, but it was possible to measure the other half of the doublet and the doublet from the methyl residues at the C 3 atom as shoulders on the large line

DISCUSSION

Ochratoxin A as a natural contaminant was first reported from U S A (*Shotwell et al* 1969) where a sample of maize was found to be contaminated with ca 150 ppb ochratoxin A By inspection of maize from different regions of U S A ochratoxin A has repeatedly been found, at levels from 83 to 166 ppb (*Shotwell et al* 1970, *Shotwell et al* 1971) This mycotoxin has also been detected in American barley samples (*Nesheim* 1971), at levels of 12–38 ppb Ochratoxin A was found in concentrations of 30 to 27000 ppb in 18 out of 29 samples of heated grain from Canadian farms (*Scott et al* 1972) The cereal

TABLE 5 *Grain Samples containing Mycotoxins (Total number assayed – 33)*

Sample no and district		Commodity	Ochratoxin A	Citrinin
			Level (ppb)	
3	I	barley	27520	0
4	I	barley	275	0
5	I	barley	2060	0
6	I	barley	138	0
7	I	barley-oats	825	0
8	I	barley	69	0
10	I	barley	69	0
11	I	barley	69	0
14	I	barley-oats	138	0
17	I	barley oats	6190	2000
18	I	barley	138	160
19	I	barley	138	0
1	II	barley	12380	1000
2	II	oats	28	0
3	II	barley	4130	0
5	II	barley	1380	0
10	II	barley oats	69	0
2	III	barley	138	0
3	III	barley	688	0
1	Control	barley	9	not assayed
2	Control	barley	189	not assayed
3	Control	barley	44	not assayed

samples consisted mainly of wheat but included also oats, barley, and rye and, in addition, ochratoxin A was detected in mouldy peanuts and dried white beans Contamination of Canadian wheat with ochratoxin A at levels of 20–100 ppb was previously reported (*Scott et al* 1970) Thirteen of the above mentioned *samples of heated* grain were simultaneously contaminated with citrinin, at levels of 70 to 80000 ppb The citrinin contaminated samples included wheat, oats, barley, and rye The association of the mycotoxin contaminated Canadian grain samples and outbreaks of disease in domestic animals was weak and no details of the pathological descriptions were recorded

The detection of ochratoxin A and citrinin in Danish samples of barley and oats corresponds well with the Canadian observations in so far as the maximal concentrations of ochratoxin A were 27520 and 27000 ppb in Danish and Canadian samples, respectively, whereas the maximal concentration of citrinin in the Canadian material (80000 ppb) was much higher than that in the Danish samples where 2000 ppb was the highest concentration observed Our observation of "double mycotoxin" contamination (Table 5), i e that citrinin always occurs together with ochratoxin A, is in full agreement with the Canadian results The high incidence of especially ochratoxin A contaminated cereal samples, in the range 50–65 per cent (Table 2), was expected because *the survey was* conducted in districts characterized by an incidence rate of mycotoxic porcine nephropathy which was 2 to 16 times higher than the mean value for Denmark in the same period of time (Table 1) In addition, the *samples* analysed were collected from farms where *cases of mycotoxic* porcine nephropathy had occurred less than one month before the date of sample collection *It appears that 200 ppb* of ochratoxin A is the lowest level at which development of porcine nephropathy is possible in the course of 4 to 5 months, i e the

normal feeding period for bacon pigs (*Krogh et al* 1973, *in preparation*)

Citrinin at levels of 200–400 mg/kg feed is required for the development of porcine nephropathy during 1–2 months (*Friis et al* 1969) Therefore, considered on the basis of the incidence of ochratoxin contaminated cereal samples (Table 2) and the cereal level (Table 4) ochratoxin A appears to be the main cause of mycotoxic porcine nephropathy, whereas citrinin seems to be a minor component

About 40 per cent of the samples collected in the districts (Table 2) did not contain ochratoxin A and citrinin, and about half of the contaminated samples contained less than 200 ppb ochratoxin A, the level required for the development of porcine nephropathy (Table 4) A possible explanation would be firstly that the sampling plan used was not designed to characterize completely the examined lots of grains Only one sample was drawn from each lot consisting of 100 to several thousands of kg A similar problem was dealt with by *Johnson et al* (1969) who analysed an aflatoxin contaminated lot of maize and found that only 64 per cent of a total of 32 samples drawn from the lot did actually contain aflatoxin Secondly, the mycoflora of grains may be able to produce other nephrotoxic mycotoxins than the two dealt with in this report Thus *Carlton et al* (1968) induced nephropathy (and liver damage) in mice by the feeding of cultures of *P viridicatum* which were unable to produce ochratoxin and citrinin (*Krogh*, unpublished)

The consequence of feeding cereals contaminated with ochratoxin A and citrinin to pigs is firstly the development of nephropathy resultating in low animal production and possible death and secondly the occurrence of residues (ochratoxin A) in organs and tissues of slaughtered bacon pigs (*Krogh & Hald*, unpublished data) similar to the occurrence of aflatoxin residues in bacon pigs (*Krogh et al* 1973) This represents a possible public health problem

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THE BALSAM OF TOLU AGGLUTINATION TEST (BTA-TEST)

I. A New Tool for Detection of Antibodies to Saline Soluble Antigen, with Special Reference to Thyroid Antibodies

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A passive agglutination technique has been evolved, using a suspension of balsam of Tolu particles as carrier for protein antigen in saline The effect of altering the variable factors in the test system is described

Jones (2) is usually credited for the introduction (in 1927) of passive agglutination techniques He showed that collodion particles sensitized with protein antigen and subsequently washed would agglutinate if small quantities of specific antibody were added

However, in 1917 already *Meinicke* (6) first described his test for syphilis which, with later modifications and improvements (7, 8), has become much used in the serological diagnosis of this disease After *Pangborn*'s (9) discovery of the cardiolipin and lecithin as the active haptens for the detection of the anti lipoidal antibodies in syphilis, these phospholipids were successfully included in the Meinicke reagent instead of crude heart extrac (4) With this kind of antigen, Meinicke s reaction is still one of the most widely employed screei ng tests for syphilis in Norway

Meinicke called the later modifications of his test "eine Klarungsreaktion" However, in the present author s opinion it is in fact, a passive agglutination test In Meinicke's extract (3) balsam of Tolu and the specific lipids are dissolved in absolute alcohol When the 3 5 per cent solution of sodium chloride is rapidly poured into the extract, a suspension of balsam of Tolu particles is obtained To these the lipids are attached If a syphilitic serum is added, the balsam particles are agglutinated by the anti lipoidal antibodies and sedimented, giving a clarification of the supernatant fluid After addition of a normal serum, the other hand, most balsam particles are kept in suspension and the fluid remains opaque Thus, while the reaction is *read* as a 'clarification test', it is [illegible] place [illegible] cent) [illegible] on of giving the solution a specific weight which, together with serum, can provide a suitable suspension stability for the unagglutinated particles

It struck the author that if the balsam of Tolu particles could carry the alcohol soluble haptens used in the standard tests for syphilis, they might also carry water soluble antigens for a passive agglutination test Working at the time on thyroid autoimmunity problems, it was decided to use thyroid extract as an antigen in the experiments

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MATERIALS AND METHODS

Balsam of Tolu is a resinous exudate from *Toluifera balsamum* The use of this balsam containing several chemical compounds in the Meinicke reagent is empirically founded The basic physicochemical properties which make it useful as an antigen carrier are not known in detail Not all batches of the balsam are suitable for this purpose We use the method deviced by *Halvorsen* (1) to check the fitness of our balsam *A 5 per cent* filtered solution of balsam of Tolu in absolute alcohol is kept as a stock solution

Another reagent needed is an 0 1 per cent solution of *Victoria blue* in absolute alcohol

Thyroid extracts were prepared from macroscopically normal human thyroid glands removed as soon as possible after death Surrounding tissues were carefully removed from the glands The glands were then finely minced with scalpels transferred to an Erlenmeyer flask and an equal *amount (w/v) of phosphate buffered isotonic saline* (pH 7 2) and a few drops of toluene were added The flask was gently swirled on a rotator overnight at 4° C The content was then centrifuged the supernatant divided in several small portions and stored at a temperature below − 22° C

The protein concentration of our extracts has ranged from about 4 to 9 g per 100 ml Such crude extracts have been found to contain about 80 per cent thyroglobulin (10) We preferred the use of crude extract to the use of purified thyroglobulin in our experiments We wanted to give any thyroid specific soluble antigen that might be present the opportunity to attach to the balsam particles

RESULTS AND DISCUSSION

Procedure of the Balsam of Tolu Agglutination Test

The solution to be used in the test contains 2 5 per cent balsam and 0 01 per cent Victoria blue and is made up e g by adding 25 ml of the balsam and 5 ml of the dye stock solutions to 20 ml of absolute alcohol The dye is added to make the results easier to read

In a large tube 10 volumes of a 2 5 per cent sodium chloride solution containing the thyroid antigen in suitable concentration (as will be discussed later) are prewarmed in a water bath at 56° C for 15 minutes One volume of the 2 5 per cent balsam solution with Victoria blue is prewarmed at the same temperature for 5 minutes in a similar tube The two solutions are mixed by briskly pouring the saline into the alcoholic solution, followed by pouring the fluid back and forth between the two tubes a few times Thereby a greyish blue suspension of balsam particles coated with thyroid antigen is obtained The mixture is kept at 56° C for another 5 minutes It is then centrifuged for 10 minutes at 3 000 rev/min and the supernatant is removed The sediment is resuspended in 2 5 per cent saline to the original volume It is necessary to shake the fluid vigorously to disperse the sediment Forcing the fluid through a pipette may also help to get a homogeneous suspension The antigen is now ready for use

In a screening test, the serum to be examined can be diluted 1 10 in isotonic phosphate buffered saline (pH 7 2) However, when an antiserum is to be titrated, we use as a medium phosphate buffered saline containing 10 per cent normal human group AB serum The addition of serum to the medium is necessary to obtain an adequate sedimentation of the antigen carrying particles

The test is performed in glass tubes with an internal diameter of about 8 to 10 mm It is important that the tubes have an evenly rounded bottom The tubes are suitably placed in transparent plastic racks To 0 1 ml of the serum dilution is added 0 1 ml of antigen suspension The racks are shaken well and placed at 4° C overnight After a half to one hour at room temperature the test is read It is important that the racks are carefully handled to avoid disturbance of the sedimentation pattern

In contrast to Meinicke's syphilis test, a sediment is formed with negative as well as with positive sera They all give a 'clarification of the supernatant The results are read according to the patterns formed by the sedimented particles The patterns are surprisingly similar to those seen in tanned cell haemagglutination tests In strongly positive reactions the particles in the periphery are folded up and give a marked wrinkle formation Occasionally strongly positive sera

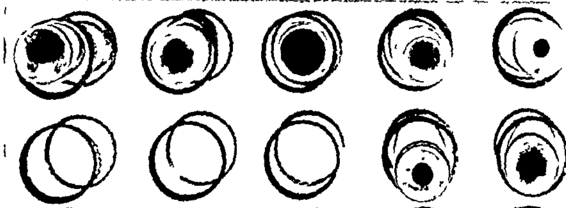


Fig 1 Sedimentation patterns of the balsam of Tolu agglutination test *Top row* Fourfold serial titration of a serum containing thyroglobulin antibodies Initial dilution 1 100
From the left Tube no 1 + + +, no 2 + +, no 3 + (+), no 4 (+), no 5 –
Bottom row Tube no 1 (left) thyroglobulin inhibition control –, tube no 2 (right) liver extract inhibition control + + +

give an even carpet of particles with no folding in the periphery As the reactions get weaker, the wrinkling becomes less pronounced Coming down to a one plus reaction, there is no wrinkling, but distinct granules are seen in the periphery In a negative reaction, finally, a relatively small button with smooth edges is formed at the bottom of the tube Sometimes, a lower concentration of particles in the centre gives the button a doughnut like appearance Some typical patterns are seen in the photograph (Fig 1)

In performing the test, we always include inhibition controls To one tube, containing 0 1 ml of the same serum dilution as the first tube in the titration series, we add 0 1 ml of a suitably diluted thyroid extract To another tube we similarly add 0 1 ml of another organ extract (usually liver or kidney) with about the same protein concentration as the thyroid extract The mixtures are incubated at room temperature for at least 15 minutes, after which 0 1 ml of the coated particle suspension is added to each tube Typically, the thyroid inhibition control will give a negative reaction, while the other tube gives a reaction of the same strength as the first tube in the titration series Now and then cases are found where the thyroid inhibition control is not negative It will then be necessary to repeat the test, this time including a thyroid inhibition control for each serum dilution in the titration series We use as a criterion for the thyroid specificity of the antibody that there must be at least an eight fold difference between the titre values of the test series and the inhibition series (i e a difference of at least 3 titration steps in a twofold serial dilution)

A 'medium control' should also be included to ensure that a clear-cut negative reaction is obtained when the antigen suspension is added to the medium alone

As stated above, the test is usually read after incubation overnight at 4° C It may, however, be kept at this temperature for two or three days without any loss in sensitivity The test may thus be performed before and read after a week end

After the first reading of the results, the tubes can be shaken well and the particles left to resediment A second reading will often show at somewhat reduced titre, but on the other hand, the end point will sometimes be more clear-cut

Effects of Variation of the Experimental Conditions

It is obvious from the description given above that a number of factors in this test

TABLE 1 *The Effect of Varying the Balsam of Tolu Concentration*

Serum dilution	g balsam of Tolu per 100 ml absolute alcohol				
	1 5	2	2 5	3	4
1 100	+ +	+ +	+ +	+ + +	+ + (+)
1 200	+ +	+ + +	+ + +	+ + +	+ + (+)
1 400	+ (+)	+ + +	+ + +	+ + +	+ + +
1 800	+	+ + +	+ + +	+ + +	+ + (+)
1 1,600	+	+ + (+)	+ + +	+ + +	+ +
1 3,200	±	+ + (+)	+ + (+)	+	+ +
1 6,400	±	+ (+)	+ (+)	+	+
1 12,800	—	±	+	±	±
1 25,600	—	—	(+)	—	—
1 51,200	—	—	(+)	—	—
1 102,400	—	—	—	—	—

Antigen Human thyroid extract no 3 diluted 1 500
Serum (no 10962) Human thyroglobulin antibody
In this experiment, the antiserum was titrated in undiluted normal human serum, and the antigen coated particles were suspended in 3 5 per cent saline, without centrifugation

TABLE 2 *The Effect of Varying the NaCl Concentration in the Antigen Suspension*

Serum dilution	Saline concentration (g per 100 ml)				
	0 5	1	2	4	6
1 500	+ +	+ + (+)	+ + (+)	+ + (+)	+ +
1 1 000	+ +	+ + (+)	+ + (+)	+ +	+ +
1 2 000	+ +	+ + (+)	+ + (+)	+ +	+ (+)
1 4,000	+ +	+ (+)	+ + (+)	+ +	+
1 8 000	+ (+)	+ (+)	+	+ (+)	±
1 16 000	*	+	+	+	—
1 32,000	*	+	(+)	(+)	—
1 64 000	*	+	(+)	—	—
1 128 000	*	+	—?	—	—
1 256 000	*	—?	—?	—	—
1 512 000	*	—?	—	—	—

Antigen Human thyroid extract no 3 diluted 1 500
Serum (no 10962) Human thyroglobulin antibody
* Atypical sedimentation (unreadable)

can be varied The effect of altering some of these factors was examined

The concentration of balsam of Tolu in absolute alcohol was varied from 1 to 4 per cent The results of one of the experiments are shown in Table 1 A 1 per cent con centration had previously been found to give no sediment under these test conditions With 1 5 per cent the particles sedimented more slowly than with the higher concentrations Otherwise there was not much variation in the results in this range of balsam concentration After repeated testing, we finally chose a concentration of 2 5 per cent as this gave nice and smooth edged negative reactions and at the same time a sensitive test, easy to read

The concentration of sodium chloride in the antigen suspension was varied from 0 5 to 10 per cent (w/v) Here a marked variation in the results occurred As seen from Table 2, an increasing saline concentration

TABLE 3 *The Effect of Varying the Antigen Concentration*

Serum dilution	Antigen dilution 1 500	1 1,000	1 2,000	1 4 000
1 100	+++	+++	+++	*
1 200	+++	+++	+++	*
1 100	++(+)	+++	+++	*
1 800	(+)	+++	+++	*
1 1 600	±	+	+++	*
1 3 200	—	±	+++	*
1 6,400	—	—	+	*
1 12 800	—	—	±	*
1 25 600	—	—	±	*
1 51,200	—	—	±	*

Antigen Human thyroid extract no 16
Serum (no 33062) Human thyroglobulin antibody
* Atypical sedimentation (unreadable)

TABLE 4 *The Effect of Washing the Antigen Coated Particles*

Serum dilution	A	B	C	D
1 500	++(+)	+++	++	++(+)
1 1 000	++(+)	++(+)	++	++
1 2 000	++(+)	++	++	++
1 4 000	++(+)	++	+(+)	++
1 8 000	+(+)	+(+)	+(+)	+
1 16 000	+(+)	+	+(+)	+
1 32 000	+	+	+	+
1 64 000	(+)	±	+	(+)
1 128 000	—	—	—	—
1 256 000	—	—	—	—

A Antigen suspension not centrifuged
B Centrifuged once sediment resuspended in the same supernatant
C Centrifuged once, sediment resuspended in pure saline
D Centrifuged twice sediment both times resuspended in pure saline
Antigen and antiserum are the same as in Table 1

gave a decreasing sensitivity of the test At the same time however, the negative reactions became nicer with a smaller and more smooth edged button Considering these conflicting results a 2 5 per cent concentration was finally chosen as the best compromise

The antigen concentration is important in all serological tests For thyroid extracts it has to be determined for each batch Among 8 different extracts prepared as described, the final dilution varied from 1 500 to 1 3 000 Correspondingly the protein concentration ranged from about 3 mg to 9 mg per 100 ml of the 2 5 per cent NaCl solution High antigen concentration gives, as expected low sensitivity of the test With falling antigen concentrations, the sensitivity increases However, at the same time the negative reactions tend to become less clear cut A certain load of protein seems to be necessary to get a particle size suitable for a good sedimentation and pattern formation (Table 3) The optimal antigen concentration is usually slightly higher than that used in a tanned cell haemagglutination test

The effect of washing the coated particles

TABLE 5 *The Effect of Storage on the Antigen Suspension*

Serum dilution	A	B	C	D	E	F
1 500	+ +	+ + (+)	+ + (+)	+ +	+ + +	+ + (+)
1 1 000	+ +	+ + (+)	+ + (+)	+ (+)	+ + +	+ + (+)
1 2 000	+ +	+ + (+)	+ + (+)	+ (+)	+ +	+ + (+)
1 4 000	+ +	+ + (+)	+ (+)	+ (+)	+ +	+ (+)
1 8 000	+ +	+ (+)	+	+	+ +	+ (+)
1 16 000	+ (+)	+	+	+	+ +	+
1 32,000	±	+	+	+	+	±
1 64 000	—?	—?	(+)	(+)	+	±
1 128 000	—?	—?	—?	—	—	—?
1 256 000	—	—	—?	—	—	—

A B C, and D The same antigen suspensions as in the experiment of Table 4 but stored for 1 month at 4° C
E Freshly made antigen suspension, not centrifuged
F Freshly made antigen suspension, centrifuged once and resuspended in pure saline
Antibody Serum no 10962

is shown in Table 4 As described above, we normally centrifuge the antigen suspension once and resuspend the sediment in pure saline The coated particles can be centrifuged once more But a second centrifugation (or too hard centrifugation) can make the resuspension of the particles more difficult. Furthermore, washing does not increase the sensitivity of the test, indicating that there is no unbound antigen left after the first centrifugation and resuspension of the particles in pure saline In fact, if the optimal amount of protein for coating the balsam particles has been carefully determined, centrifugation can be omitted without any reduction in sensitivity This shows that the binding of the antigen to the balsam of Tolu particles is very firm

The effect of storage on the antigen suspensions is demonstrated in Table 5 Four batches of coated particles, ready for use, were stored for 1 month at 4° C The suspensions were then vigorously shaken and immediately added to titration series of an antiserum, in parallel with two freshly made antigen suspensions The stored suspensions proved to give as good results as the fresh ones This also seems to corroborate the firm binding of the thyroid antigen to the balsam

The influence of the medium used for titration of the antisera was examined Different concentrations of normal human serum in phosphate-buffered saline were compared A 10 per cent serum concentration seemed to give the best results, as judged from the negative controls and positive reactions together Perhaps more important than the exact serum concentration is the selection of a suitable donor of the serum to be used in the medium One must, of course, be sure that the serum contains no antibody against the antigen extract used However, after this has been ruled out, it is found that some sera, for reasons unknown, do not give sufficiently clear-cut negative reactions The factor(s) responsible for this phenomenon seem(s) to occur more frequently in sera from female than from male donors

Limited testing of one per cent normal rabbit serum in isotonic phosphate buffered saline gives some indication that this also could be used as a medium In principle, however, it is preferable to avoid the introduction of heterologous serum in the test system The author is, of course, fully aware of the usefulness of diluted rabbit serum as a medium in the tanned cell haemagglutination test

Different incubation temperatures were compared The tests were kept at 4° C, room temperature, and 37° C The latter temperature gave the least sensitive reactions In-

cubation at 4° C and at room temperature gave relatively similar results, those at 4° C perhaps being slightly better

The usefulness of a serological test depends on several factors Of primary importance are the specificity, the sensitivity, and the reproducibility of the reactions The applicability and the simplicity in performance also play a role for the value of a test The results obtained with the present test in examinations for thyroid antibodies will be the subject of a subsequent report (5)

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INDUCTION AND SURFACE GROWTH OF L-PHASE VARIANTS OF DIFFERENT *ESCHERICHIA COLI* STRAINS

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L phase variants of *E coli* show a great strain variability as regards their capacity to grow on agar surfaces Using two different media containing 1000 IU penicillin/ml, L phase induction was attempted with 61 *E coli* strains of different serological types The use of 10,000 and 50,000 IU penicillin/ml was less favourable Forty seven strains were subcultured With 32 strains, no or a single subcultivation was possible With 9 strains, abundant surface growth of L-colonies was obtained after four subcultivations or more With three strains, more than 90 serial transfers were performed on penicillin-free media

L-phase growth in *E coli* is more capricious and strain-dependant than in other members of the *enterobacteriaceae* group such as *Proteus* and *Salmonella* (3, 8, 11, 14) Surface growth of *E coli* L-phase variants has only been obtained with certain strains (3, 13)

Lederberg & *St Clair* reported that the yield of L-colonies was dependent on strains and substrains (11) Using different serum-free media, the authors also studied the growth of non-stable *E coli* L-colonies in pour plates Using a serum-containing medium, *Dienes* & *Sharp* reported that 5 strains of coliform organisms produced large L-colonies as surface growth in the presence of penicillin, and one of these strains was subcultivated 4 times (3) Later, *Schumann* & *Taubeneck* obtained stable *E coli* L-phase variants from certain strains on serum-containing media (13)

The aim of the present investigation was to study *E coli* strains of different serological types in order to find strains with a high capacity to grow as L-colonies on the surface af agar media

L-phase variants are cell wall defective bacteria capable of reproduction On suitable solid media they form distinctive colonies known as L-colonies (3, 9, 12)

Received 2 iv 73 Accepted 29 vii 73

MATERIALS AND METHODS

E coli strain[illegible]
was the test [illegible]
60 were the [illegible]
with exceptı[illegible]
from the WHO International Escherichia Centre, Statens Seruminstitut, Copenhagen, Denmark

Culture media Two different solid media were used in parallel throughout the study One medium, here called the CA-medium, was similar to the medium described by *Conner et al* (2) The other medium, called the TY-medium, was based upon the TY-1 medium for cultures of *E coli* in shaken flasks (5) and has been described earlier (7)

CA-medium (per 1000 ml) sucrose, 100 g, K_2HPO_4, 16 g, KH_2PO_4, 2 g, Na citrate $2H_2O$, 0 82 g, $MgSO_4$ $7H_2O$, 0 2 g, nicotinic acid, 35 mg, vitamin-free casamino acids (Difco), 10 g, agar (Bacto, Difco), 10 g, horse serum, inactivated at 56° C for 30 min, 100 ml

TY medium (per 1000 ml) sucrose, 100 g, glucose, 10 g, $MgSO_4 \cdot 7H_2O$, 0 2 g, tryptone (Difco), 10 g, yeast extract (Difco), 5 g NH_4Cl, 2 5 g, $Na_2HPO_4 \cdot 2H_2O$, 15 g, KH_2PO_4, 6 g, $Na_2SO_4 \cdot 10H_2O$, 0 5 g, agar Noble (Difco), 10 g, horse serum, inactivated at 56° C for 30 min, 100 ml

Both media were used with addition of 1000 IU penicillin per ml unless otherwise stated The TY medium was also used in liquid form without agar for growth of the bacterial inocula and in some cases in the serial transfers of L colonies

Induction and transfer of L-colonies The *E coli* strains were grown in liquid TY medium in static tubes for 4 hours and 0 1 ml was spread over the solid media The plates were incubated anaerobically during 4 days at 37° C, followed by 3 days' incubation in 5 per cent CO_2 in air In the case of some strains, parallel plates were incubated aerobically from the first day Subcultivations of L colonies were made with the agar to agar technique (10) The plates were inspected on day 4, 6 and 7 for macroscopic growth, using an inverted microscope

MIC values for penicillin were determined with a paper disc method (4) using 500 and 10 mcg discs

RESULTS

Broth cultures were inoculated onto the penicillin-containing agar media All strains but one showed macroscopically visible growth after 4 days' incubation The surface growth consisted of three types of colonies 1) classical L colonies with a dense centre and a vacuolated periphery, and/or 2) homogenous colonies lacking the distinctive centre but otherwise fulfilling the criteria for L-colonies (L-phase variants) and/or 3) classical bacilliform growth

Serial transfers were attempted with 47 of the strains With 32 strains a single or no subculture was possible Six strains could be transferred twice and nine strains could be subcultivated four times or more (Table 1) Three of these strains O2 O8 and O15, were subcultivated further After 2–4 serial transfers on penicillin containing plates the strains could be cultivated on penicillin-free media At present these strains have been subcultivated about 100 times Among all the investigated strains the strain O8 showed the most abundant growth consisting of 4–5 mm large L-colonies The stable L-colonies of this strain showed increasing slime production after repeated subcultivations

TABLE 1 *The 9 Strains with High Productivity for L phase Variants which Were Found among 61 Investigated E coli Strains*

Strains	O antigen no	No of subcultivations performed
U9/41	2	>90
G3404/41	8	>90
Bi626/42	12	4
F7902/41	15	>90
F10018/41	18	4
F9884/41	27	4
E40	33	4
H710c	41	4
U19/41	51	4

Weekly subcultivations were necessary with all strains, subcultivations after 12 days were, as a rule, not successful The L-colonies of the nine strains shown in Table 1 consisted of colonies with a dense central area of growth, whereas the other strains lacked the dense centre of the colony Furthermore, these nine strains showed the highest induction frequency and the heaviest surface growth of all the investigated strains After inoculation onto penicillin-containing agar plates, the above mentioned nine strains showed no or very slight bacilliform growth compared to other strains with similar MIC-values for penicillin Absence of bacilliform colonies, however, was also found among strains with low MIC-values for penicillin such as the strains O5 O11 and O25 whose ability to grow as L phase variants was poor

Anaerobic incubation was superior during primary induction with most strains but not during the following subcultivations When growth as L-phase variant had been established in a strain, aerobic and anaerobic incubation gave similar results The L-colonies had a more distinctive appearance on the CA-medium than on the TY-medium In other respects, the two media were similar

L phase induction was attempted with 20

strains, O1–O20, on the TY medium containing 1000, 10,000 and 50,000 IU of penicillin G/ml Strain O8 was the only strain that produced classical L-colonies of the "fried egg" type both with 1000 and 10,000 IU/ml Classical L-colonies of the "fried egg"-type were produced by the strains O2, O12, O15 and O18 with 1000 but not with 10,000 or 50,000 IU/ml In addition, these strains produced L colonies of the homogenous type lacking the dense central area with 1000 IU/ml, and less abundantly with 10,000 IU/ml With 50,000 IU penicillin/ml almost no surface growth could be observed

The reproducibility of the results was strain dependent The strain O8 gave very constant results, both as regards induction of L-colonies and serial transfers Higher yields of L colonies after subcultivation were obtained if the plate was flooded with TY-medium before the agar to agar transfer L-colonies were also serially transferred by shaking agar blocks in 2–5 ml TY-medium and inoculating 0 5–1 ml of the medium onto agar With this method for serial transfers the agar surface became evenly inoculated

DISCUSSION

Abundant growth of stable *E coli* L colonies on agar surfaces offers a simple method for the harvest of large quantities of L phase variants for immunological and other studies (6, 7) With the present methods and media, however, only a certain proportion of *E coli* strains (9 out of 47) could be subcultivated four times or more This is in accordance with *Artemieva* who found that 48 out of 225 investigated enteropathogenic *E coli* strains produced "L forms (1) The reason for this strain variability as regards L-colony production in *E coli* is not known (11, 13)

Certain *E coli* strains have been shown to require concentrations of penicillin higher than 1000 IU/ml for the induction of L-colonies (11, 13) However, among the present 20 investigated strains, strain O8 was the only strain that produced L-colonies in the presence of 10,000 IU penicillin/ml These colonies were morphologically similar to those produced by 1000 IU/ml

During induction, two characteristics could be used independently to find strains with a high productivity for L phase variants, namely the abundance of the surface growth and the morphology of the colonies During the present conditions, all strains with a high capacity for L-phase growth grew as typical L-colonies of the "fried egg"-type However, stable *E coli* L-phase variants without the "fried egg" appearance have been described (13) The nine strains with high productivity for L phase variants may have been favoured by the present cultural conditions Studies in progress have shown that certain environmental factors profoundly affect the ability of individual *E coli* strains to produce L-phase variants

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The excellent technical assistance of Mrs. *Ingrid Wargskog* is gratefully acknowledged

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Acta path microbiol scand Section B 81, 707–710, 1973

AN EVALUATION OF THE EFFECTS OF A HIGH CONCENTRATION OF SUCROSE IN BLOOD CULTURE MEDIA

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During 6 months a trial was run on the effect of the addition of 10 per cent sucrose to blood culture media The standard procedure for blood culturing is to inoculate the sample into 12 individual tubes, and the trial was performed by adding 10 per cent sucrose to half of the tubes and then comparing the results obtained in the tubes with and without sucrose The overall isolation rate of microorganisms was not affected but the detailed comparison showed that for nearly all species the sucrose containing media tended to yield growth more frequently than the media without sucrose This difference was statistically significant for enterobacteria and *Staphylococcus aureus*

The present study was prompted by the finding by *Rosner* that sucrose significantly improves the results of blood culturing (4) As the methods in use in this laboratory are different from those of *Rosner*, it was considered important to find out whether the addition of sucrose to our media would also improve the results of our blood cultures

MATERIALS AND METHODS

The Department of Diagnostic Bacteriology at Statens Seruminstitut in Copenhagen receives blood samples for bacterial culture from most Danish hospitals all over the country The present material consists of 10,717 blood samples received in the period July 1 to December 31, 1970

A maximum of 8 ml of blood is drawn into a Venule® which consists of an evacuated glass tube containing 1 ml of a 1 per cent solution of sodium polyanetholsulphonate (Liquoid) The blood is transported to the laboratory in the venule and upon arrival there it is distributed as evenly as possible—in the order described below—by the aid of four Pasteur pipettes into 12 tubes of culture media arranged in a rack as follows Four tubes of nutrient broth (filtered ox broth with 5 per cent ox serum, 0 1 per cent glucose and 0 35 per cent haemolysed horse blood), four tubes of semisolid nutrient agar (filtered ox broth with 0 2 per cent Bacto agar (Difco)) and four tubes of semisolid thioglycollate agar (filtered ox broth with 0 3 per cent thioglycollate, 5 per cent pepsin digested horse blood and 0 2 per cent Bacto agar (Difco)) With the first pipette the No 1 tube of each of the three media is inoculated, the No 2 tube of each medium is thereafter inoculated with the second pipette, and so on This procedure was originally introduced by *Jorgensen* (2) and later modified several times (7, 8) During the trial period the No 1 and the No 2 tubes of each medium contained sucrose in a concentration of 10 per cent The time interval between blood sampling and inoculation varied roughly from 1 hour to 24 hours The inoculated tubes were incubated at 35° C and inspected for signs of macroscopically visible growth twice daily during the first 2 days and thereafter once daily for a further 5 days Tubes showing signs of growth were examined under the microscope and sub-

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cultured on relevant plate media The isolated bacterial strains were identified according to the routine procedures of the laboratory

For each positive blood sample, the number of tubes showing growth was recorded separately for tubes with and without sucrose In order to facilitate the analysis, only those samples where the amount of blood received had been sufficient to inoculate all 12 tubes were included in the calculations Samples giving rise to growth of more than one bacterial species were excluded from the analysis, as were obvious contaminants Thus, *e g*, *Escherichia coli* was isolated from 95 samples which had been inoculated into less than the maximum 12 tubes, and these, together with 48 samples which grew *E coli* in mixture with one or more other bacterial species, were excluded, leaving a total of 233 positive samples However, the final analysis was limited to a comparison between the number of No 2 tubes (containing sucrose) showing growth and the number of No 3 tubes (not containing sucrose) showing growth This was done because it was realized that if less blood than usual was available there would be a risk of having inoculated a smaller amount of blood into the No 4 tube of each medium than into the other tubes

For the analysis, the samples giving rise to growth were divided into three classes 1) those in which growth occurred in more tubes with sucrose than in tubes without sucrose, 2) those in which growth occurred in fewer tubes with sucrose than in tubes without sucrose, and 3) those in which growth occurred with equal frequency in tubes with and without sucrose

The results were compiled and analysed separately for each of the most commonly encountered bacterial species As an example, the data of the 122 blood samples, *included in the final analysis* which yielded growth of *Escherichia coli* are shown in Fig 1 The figures appearing below the diagonal represent the number of samples constituting class 1, 63 in all, the figures appearing above the diagonal represent the number of samples constituting class 2, 40 in all, and the figures lying on the diagonal represent the samples constituting *class 3, 19 in all*

The classes were compared on the assumption that if sucroce had not had any influence on the rate of isolation of bacteria from blood samples, then the number of samples in class 1 should display a binominal distribution with $p = \frac{1}{2}$ and n equal to the total number of samples in class 1 plus class 2, and P values were calculated according to this hypothesis using Yates correction and a one tail test* In other words the calculations were based on the assumption that if sucrose had not had any effect, the number of samples in class 1 and class 2 should be almost the same

A comparison was also made between the total number of blood samples giving rise to growth expressed as the percentage of the total number of samples examined during the trial period and the corresponding figure from the same period of the year 1969

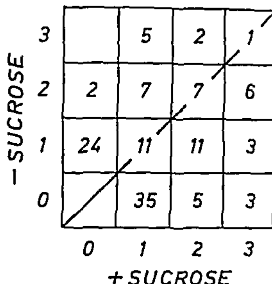


Fig 1 Blood samples giving rise to growth of *Escherichia coli* in the No 2 and the No 3 tubes of the three media, with and without sucrose, respectively The horizontal scale represents the number of tubes containing sucrose with growth and the vertical scale represents the number of tubes not containing sucrose with growth The figures in the diagram represent number of samples

RESULTS

During the trial period altogether 10,717 blood samples were cultured and 1,392 of these yielded growth Thus, the overall isolation rate was 13 0 per cent in comparison with 14 0 per cent during the same period of the year 1969 when sucrose was not added to any of the tubes In 1965 and 1971, the corresponding figures covering the whole year were found to be 13 2 per cent and 13 6 per cent, *respectively (1)* The difference between these four isolation rates is insignificant and it may therefore be concluded that the addition of sucrose in a concentration of 10 per cent did not influence the overall isolation rate in this study

* The calculations were carried out by *S Olesen Larsen*, M A, Biostatistical Department, Statens Seruminstitut

TABLE 1 *Distribution of the Isolates of Different Microorganisms into Class 1 and Class 2*§

Organism	Class 1†	Class 2
Escherichia coli	63*	40
Klebsiella spp	10	7
Proteus mirabilis and *vulgaris*	10*	3
Other enterobacteria	7	7
All enterobacteria	90**	57
Pseudomonas aeruginosa	8	3
Staphylococcus albus	30	27
Staphylococcus aureus	31*	18
Streptococci	10	8
Bacteroides spp	3	5

§ Class 1 Samples where number of positive No 2 tubes containing sucrose > number of positive No 3 tubes not containing sucrose
Class 2 Samples where number of positive No 2 tubes < number of positive No 3 tubes
† Significance level class 1 versus class 2
* $P < 0.05$,
** $P < 0.01$,
remainder not significant

Table 1 shows for each of the most commonly encountered species, or species groups, the distribution of the samples in class 1 and class 2 based on the registration of growth in the No 2 and the No 3 tubes It is apparent from the table that class 1 is larger than class 2 for all the bacteria listed with the exception of the small groups "other enterobacteria" and *Bacteroides* spp In some cases (marked in the table) the difference is statistically significant, this together with the general trend, permits the conclusion that the addition of 10 per cent sucrose does enhance the frequency with which growth of bacteria is obtained in the primary blood culture media If the comparison was made between all tubes containing sucrose with growth and all tubes not containing sucrose with growth the difference was found to be even more pronounced, but, as mentioned previously, this could be partly due to a smaller amount of blood having been inoculated into the No 4 tubes than into the other tubes

In the analysis comprising all tubes it was found that out of the 233 samples with *E coli*, 118 gave rise to growth in less than the maximum 12 tubes indicating that more than 50 per cent of the samples contained less than 12 viable units per 8 ml, *i e*, roughly one viable unit per ml Furthermore, 55 samples gave rise to growth in only one tube, *i e*, more than 20 per cent of the samples contained less than one viable unit per ml In other words, only a very low number of viable bacteria are recovered from a surprisingly high number of cases of bacteriaemia A fact which explains why it was necessary to consider the influence of the size of the inoculum

In the case of *E coli*, the number of samples permits an analysis of the ratio between the number of samples giving rise to growth exclusively in the No 2 tubes with sucrose and the number of samples giving rise to growth exclusively in the No 3 tubes without sucrose From Fig 1 it can be seen that in 43 cases *E coli* occurred only in tubes containing sucrose and in 26 cases only in tubes without sucrose This difference is significant ($P < 0.05$) Such differences are not reflected in the total isolation rate because only a small fraction of samples yields growth exclusively in either one or the other kind of tubes Most will, of course, yield growth in both kinds of tubes

Incidentally, the effect of sucrose on the isolation of *E coli* could not be correlated to the presence or absence of the ability of the strains to ferment sucrose

Although not subject to a systematic study, it was the general impression of those involved in the daily work with the blood cultures that the tubes containing sucrose very often showed visible growth several hours before the tubes without sucrose In such cases microscopy revealed a high proportion of aberrant morphological forms

Unfortunately, the isolates of *Neisseria meningitidis* and *Haemophilus influenzae* obtained during the trial period cannot be analysed because all samples either grew in all tubes or were inoculated into less than 12 tubes and therefore excluded Another recent study from this laboratory, however, revealed a statistically significant increase in the number of isolates of these two bacterial

species in 1971 as compared to 1965 This might, at least partly, be due to an effect of the addition of sucrose to the media in 1971 (1)

DISCUSSION

Practically all the bacterial species covered by the analysis exhibited *growth in* a larger number of tubes with than without sucrose As far as three of the nine species, or species groups, analysed are concerned, this difference was statistically significant, although it did not result in a higher overall isolation rate in comparison with that of the same period of the year before when none of the blood culture tubes contained sucrose

Rosner, in 1970, reported an increase in the recovery of bacteria from the blood if 10 per cent sucrose was added to the culture medium already containing Liquoid (4) Corresponding results were obtained by others Thus, *Sullivan et al* (6) added 16 per cent sucrose and magnesium sulphate to an anaerobic broth containing Liquoid, and *Wolfe & Amsterdam* (9) used a thioglycollate medium with 20 per cent sucrose In this latter study it was observed that the initial growth consisted of pleomorphic organisms

Recently, *Rosner* (5) has confirmed and extended his original observations He found that gram negative enteric bacteria grew equally well *in all of the test media* According to this study, the effect of sucrose was most marked on the following organisms, *viz* *Bacteroides* spp , anaerobic streptococci, *Neisseria meningitidis* and *Haemophilus* spp Furthermore he discusses the mechanism of the effect of sucrose and concludes that it does not act as a growth stimulant, but rather offers protection to cell wall damaged bacteria from osmotic pressure changes, thereby possibly reducing the lag phase of at least some cells It appears reasonable to assume, as *Rosner* and *Sullivan et al* do (5, 6), that some of the bacteria in the circulating blood of a bacteraemic patient exhibit some degree of cell wall damage and that osmotic stabilization will protect and save some of the damaged cells and shorten the time needed for resynthesization of defective cell wall material prior to normal cell growth *In vitro* studies have unequivocally demonstrated the existence in human serum of an antibody complement system lethal to L phase variants of certain bacteria (3), and therefore it can be assumed that sucrose will be able to protect only cells with less than total cell wall damage

In this study, greatly varying periods of time elapsed before the blood samples were inoculated into the media, and this might explain why we, in contrast to *Rosner*, also find an effect of sucrose on the isolation of *E coli*

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DNA REPAIR SYNTHESIS IN MOUSE P-388 CELLS TREATED WITH MITOMYCIN C

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DNA repair synthesis was studied in mitomycin C treated mouse P 388 cells grown in suspension culture by measuring the incorporation of ^{3}H thymidine into non replicating DNA Two methods of density labelling with bromodeoxyuridine were used By the first method, repair synthesis was measured as incorporation of ^{3}H thymidine into light DNA (banding at 1 70 in neutral CsCl) during simultaneous labelling with ^{3}H thymidine and bromodeoxyuridine Increasing amounts of ^{3}H labelled light DNA were then found after exposure to increasing concentrations of mitomycin C By the second method, repair synthesis was measured as incorporation of ^{3}H thymidine into hybrid DNA (banding at 1 75 in neutral CsCl) of cells grown in medium containing bromodeoxyuridine prior to treatment with mitomycin C Exposure to increasing concentrations of the antibiotic then resulted in a progressive accumulation of ^{3}H labelled hybrid DNA The results indicated that the mouse P 388 cells carried out repair of DNA lesions after treatment with mitomycin C

The cytotoxic effect of mitomycin C is believed to depend on its ability to alkylate DNA (*Szybalski & Iyer* 1967), and it has been suggested that recovery of mammalian cells after treatment with the antibiotic may be due to repair of DNA (*Rauth et al* 1970)

In previous work from our laboratory (*Ørstavik* 1972 a, b, c) data were presented indicating that mouse P 388 cells may possess mechanisms for the repair of mitomycin C induced damage More detailed experiments on repair phenomena in these cells following treatment with mitomycin C are reported in this paper The results suggest that recovery of the P-388 cells after exposure to mitomycin C may be related to repair of DNA lesions

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MATERIALS AND METHODS

Chemicals

Mitomycin C (Kyowa Hakko Kogyo Co Ltd, Tokyo) was kept as aqueous stock solution (1 mg/ml) at +4° C and used within one week 5 bromodeoxyuridine (Nutritional Biochemicals Corporation) was dissolved in water (250 µg/ml) and sterilized by filtration Thymidine 6 T (n), (> 15 Ci/m mol) and thymidine 2 ^{14}C, (> 50 mCi/m mol) were obtained as sterilized aqueous solutions from The Radiochemical Center, Amersham. The radioactive compounds were stored at +4° C for maximum 2 months Cesium chloride (analytical grade) was purchased from British Drug Houses, and Insta Gel® scintillation liquid from Packard Instrument Co Inc

Cell Cultures

P 388 mouse cells (*Dawe & Potter* 1957) were grown in suspension culture on Eagle's minimum essential medium containing 10 per cent foetal bovine serum as described previously (*Ørstavik* 1972 a)

Isolation of DNA

Approximately 10^7 cells were chilled at 0° C, harvested by centrifugation, washed in SSC* at 0° C and suspended in the same buffer (approximately 4×10^9 cells/ml) Cell lysis was obtained by adding 0 2 ml of 11 per cent (w/v) sodium lauryl sarcosinate containing 5 mg/ml of naphtalene-1 5 disulphonate to 2 ml cell suspension The lysate was deproteinized twice by shaking with phenol (½ volume) ,and centrifugation at +4° C to separate the phases Two volumes of 96 per cent ethanol were added to the aqueous phase and the nucleic acids were precipitated at −20° C overnight The precipitate was washed twice in ethanol /water/sodium acetate (300 ml/ 100 ml/ 8 g) and finally dissolved in 3 ml of dilute SSC (1 10) The content of DNA was measured as diphenylamine-reacting material (*Burton* 1956)

Isopyknic CsCl Centrifugation

The nucleic acid preparation was subjected to buoyant density centrifugation in neutral CsCl (CsCl in dilute SSC, pH 7 2) The first banding was carried out in preformed two step gradients to obtain rapid equilibrium (*Brunk & Leick* 1969), 30–50 μg of DNA uniformly mixed in 3 ml of CsCl (final density 1 60) was carefully layered on top of 3 ml of CsCl with a density of 1 84 The tubes were topped with paraffin oil and centrifuged in a Beckman Spinco 50Ti rotor at 30 35000 rev/min and +25° C for 16–20 hours Fractions (200–250ul) were collected from the bottom of the tubes and analysed Rebanding was performed by pooling selected fractions and adding CsCl and SSC to a final volume of 6 ml and a density of 1 70–1 72 followed by ultracentrifugation for 3 days The gradients were fractionated as above

The density of the fractions was measured in an Abbe s refractometer (*Szybalski* 1968)

Radioactivity Assays

After the first banding in CsCl 25 μl from each fraction was diluted with 0 6 ml of distilled water and dissolved in 10 ml of Insta-Gel® scintillation liquid

After the second banding carrier DNA (250 μg) was added to each fraction followed by precipitation with 4 ml of ice cold TCA (10 per cent w/v) for 20 minutes The p ecipitates were washed with 3 ml 75 per cent ethanol/diethyl ether (1 1) and dried Hyamine hydroxide (250 μl) was added and, after heating at +70° C for 10 minutes, the solution was transferred to vials containing 10 ml of scintillation liquid (toluene containing 4 g/l of PPO, 0 05 g/l of dimethyl POPOP and 3 ml/l of concentrated acetic acid)

Counting was performed in a Packard Tri Carb model 3365 liquid scintillation counter, and in doubly labelled samples dpm-^{3}H and dpm ^{14}C were calculated (*Paus* 1973)

Repair Synthesis Experiments

Two methods were used for the determination of DNA repair synthesis in P 388 cells treated with mitomycin C Both methods were based on density labelling with bromodeoxyuridine essentially as described by *Roberts et al* (1968) The density labelling schemes as modified for the P 388 cells have been outlined in Fig 1

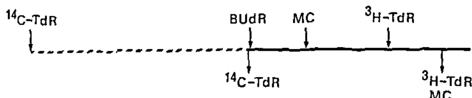
In scheme I, cells were grown from an initial density of 5×10^5 cells/ml for 8 hours in the presence of ^{14}C thymidine (7×10^{-8}M, 0 005 μCi/ml) The cells were collected by centrifugation, washed in medium and suspended at a density of 2×10^6 cells/ml in medium containing bromodeoxyuridine (5 μg/ml) Further treatment was carried out in the presence of this heavy DNA precursor The cells were first grown in the presence of bromodeoxyuridine alone for 2 hours Mitomycin C was then added and incubation continued for 3 hours Then the cultures were supplemented with ^{3}H thymidine (2×10^{-7}M, 4 5 μCi/ml) and incubation with this radioactive precursor (still in the presence of bromodeoxyuridine and antibiotic) continued for 2 hours The cells were collected by centrifugation suspended at the same density in medium containing bromodeoxyuridine alone (5 μg/ml) and finally incubated for 1 hour before they were harvested

In scheme II, cells were grown from an initial density of 5×10^5 cells/ml for 6 hours in the presence of bromodeoxyuridine (5 μg/ml) The cells were collected by centrifugation washed in medium and suspended in normal growth medium at a density of 10^6 cells/ml After incubation for 1 hour the cultures were supplemented with mitomycin C and again incubated for 1 hour The cells were then collected by centrifugation washed in medium resuspended to a cell density of 2×10^6/ml in medium containing ^{3}H thymidine (2×10^{-7} M, 5 μCi/ml) incubated for 3 hours and harvested

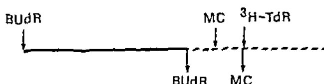
DNA was isolated and subjected to neutral CsCl gradient centrifugation as described Repair synthesis in cells treated according to scheme I was measured as incorporation of ^{3}H thymidine into DNA of normal density The other method (scheme II) relied upon the incorporation of ^{3}H thymidine

* Abbreviations MC mitomycin C SSC sodium chloride (0 15 M) and tri sodium citrate (0 02 M) pH 7 2 TCA trichloroacetic acid TdR thymidine BUdR 5 bromodeoxyuridine PPO 2 5 diphenyloxazole dimethyl POPOP 1 4 bis-2 (4 methyl 5 phenyloxazolyl benzene rev /min revolutions per minute dpm disintegrations per minute, cpm counts per minute UV ultraviolet

SCHEME I



SCHEME II



-10 -8 -6 -4 -2 0 2 4 6

HOURS

Fig. 1 Density labelling schemes by which to distinguish between replicating and non replicating DNA in mitomycin C treated P 388 cells.

Scheme I consisted of the following steps. 1. Incubation with ^{14}C thymidine (8 hours) for reference labelling of light DNA. Further treatment was carried out in medium containing bromodeoxyuridine. 2. Incubation with bromodeoxyuridine alone (2 hours) to start density labelling of replicating DNA. 3. Exposure to mitomycin C (3 hours). 4. Labelling with ^{3}H thymidine (2 hours with mitomycin C still present). 5. Incubation with bromodeoxyuridine (1 hour) to complete density labelling of replicating DNA.

Scheme II consisted of the following steps. 1. Incubation with bromodeoxyuridine (6 hours) for density labelling of part of the DNA in the culture. Further treatment was carried out in medium without heavy precursor. 2. Incubation in normal growth medium (1 hour) to start replication in the absence of bromodeoxyuridine. 3. Exposure to mitomycin C (1 hour). 4. Labelling with ^{3}H thymidine (3 hours).

For experimental details see text.

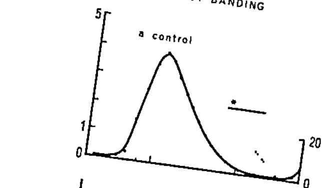
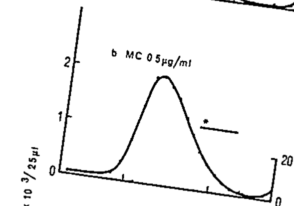
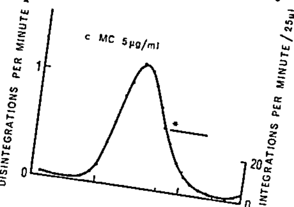
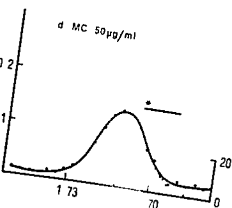
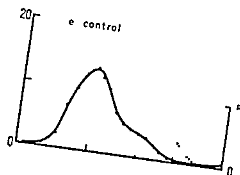
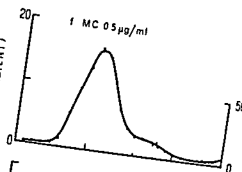
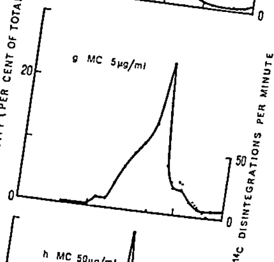
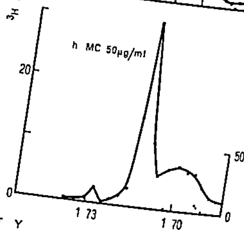
into density labelled DNA as an indicator of repair synthesis.

RESULTS

Introductory experiments showed that DNA from P-388 cells grown in medium without BUdR banded at a density of 1.70. If the medium contained BUdR, DNA with higher buoyant densities was synthesized. With BUdR alone (5 μg/ml) the density was approximately 1.75, with BUdR (5μg/ml) and TdR (2×10^{-7}M) it was slightly below 1.73. BUdR-substituted DNA will be referred to as hybrid in the text, DNA with a normal density (1.70) will be referred to as light.

DNA Repair Synthesis in Mitomycin C Treated Cells

In the first set of experiments, treatment with mitomycin C and subsequent labeling with ^{3}H TdR was carried out in medium containing BUdR (Fig. 1, Scheme I). Semi conservatively replicated DNA should then acquire hybrid density and also be ^{3}H labelled. On the other hand, a possible repair synthesis in the non replicating parts of the


FIRST BANDING
a control
5
1
0
20
0

b MC 0 5μg/ml
2
1
0
20
0
DISINTEGRATIONS PER MINUTE x 10 3/25μl

c MC 5μg/ml
1
0
20
0
14C DISINTEGRATIONS PER MINUTE /25μl

d MC 50μg/ml
1 73
70
20
0
D E N S I T Y

REBANDING
e control
20
0
0

f MC 0 5μg/ml
20
0
0
3H RADIOACTIVITY (PER CENT OF TOTAL IN THE GRADIENT)

g MC 5μg/ml
20
0
50
0
14C DISINTEGRATIONS PER MINUTE

h MC 50μg/ml
20
0
50
0
1 73
1 70

DNA while leading to some incorporation of ^{3}H TdR should not involve sufficient in corporation of BUdR to increase the density of these DNA regions appreciably (*Cleaver* 1969)

Fig 2 shows the density profiles of ^{3}H labelled DNA from control cells and cells treated with increasing concentrations of mitomycin C during two consequtive band ings in CsCl It appears that exposure to mitomycin C caused an accumulation of ^{3}H labelled DNA in the 'light' region of the gradients (Fig 2, right column) This finding suggests that the treatment had induced DNA repair synthesis in non replicating DNA of the P-388 cells A rough quantitative estimate of this effect of mitomycin C was obtained by calculating the ratio of dpm-^{3}H / dpm ^{14}C in the fractions containing the ^{14}C-labelled light DNA after rebanding The ratio increased with increasing concentrations of the antibiotic control, 1 8, MC 0 5 μg/ml, 2 0, MC 5 μg/ml, 3 7 and MC 50 μg/ml, 14 1

It should be noted that, in these experi ments, increasing concentrations of mitomycin C caused a progressive shift of the main band of ^{3}H labelled DNA towards the light part of the gradient (Fig 2, left co lumn) The displacement of the main band might contribute to the occurrence of ^{3}H radioactivity in the 1 70 region of the reband ed gradients, and thus interfere with the interpretation of the latter finding

Therefore, DNA repair synthesis was also measured by another method In these experiments the cells were first grown for 6 hours in medium containing BUdR and then transferred to medium without BUdR before the treatment with mitomycin C started (Fig 1, Scheme II) These cells contained both hybrid and light DNA during labelling with ^{3}H-TdR. Apparently, the hybrid part of DNA did not participate in semiconservative replication, since all ^{3}H labelled DNA from cells not treated with mitomycin C banded at a density of 1 70 (Fig 3 a and e) Thus, by this method, incorporation of ^{3}H-TdR into hybrid DNA may serve as a measure of DNA repair synthesis

The results presented in Fig 3 (right co lumn) show that treatment with mitomycin C lead to the appearance of ^{3}H labelled DNA banding at hybrid density (above 1 73), indicating that repair synthesis occurred The extent of repair synthesis appeared to be dependent on the concentration of mitomycin C Thus, the total amount of ^{3}H radio activity found in the hybrid section of the gradients after rebanding increased with in creasing concentrations of the antibiotic control, 97 cpm, MC 0 5 μg/ml, 114 cpm MC 5 μg/ml 252 cpm and MC 50 μg/ml, 352 cpm

DISCUSSION

We have previously shown that after a short exposure to 0 5 μg/ml of mitomycin C DNA synthesis and cell growth was only temporarily suppressed in mouse P-388 cell cultures (*Ørstavik* 1972 a, b, c) These observations are compatible with the view that mam malian cells may possess a mechanism for the repair of mitomycin C induced lesions in DNA (*Rauth et al* 1970) The present work confirms this view Thus the data obtained by two different methods indicated that the P 388 cells carried out DNA repair syn thesis after treatment with mitomycin C (Fig 2 and Fig 3)

Simultaneous labelling with heavy and radioactive DNA precursors (Scheme I in the present study) is the method most ex tensively used to demonstrate DNA repair synthesis in mammalian cells (e g *Rasmussen & Painter* 1966, *Painter & Cleaver* 1967,

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Fig 2 Density profiles of DNA from P 388 cells treated with mitomycin C and labelled with bro modeoxyurid ne and ^{3}H thymidine (scheme I) ^{14}C radioactivity profile serves as a reference for DNA of normal density (dotted line)

Note change of scale for ^{3}H radioactivity after the first banding.

* Fractions selected for rebanding

Bandings in neutral CsCl carried out at 30 000 rev/min

FIRST BANDING

REBANDING

a control

e control

b MC 0 5μg/ml

f MC 0 5μg/ml

c MC 5μg/ml

g MC 5μg/ml

d MC 50μg/ml

h MC 50μg/ml

^3H COUNTS PER MINUTE x 10^{-3}/ 25μl

^3H RADIOACTIVITY (PER CENT OF TOTAL IN THE GRADIENT)

1 75 1 70

1 75 1 70

D E N S I T Y

Roberts et al 1968) In the present study ambiguous results were obtained by this method It appeared that an effect of mitomycin C on DNA replication might also contribute to the formation of ³H-labelled light DNA Thus, in mitomycin C treated cells the density of the semiconservatively replicated DNA was reduced (Fig 2, left column) This finding probably reflects low incorporation of BUdR due to inhibition of DNA replication (*Ørstavik* 1972 b) Semiconservatively replicated DNA with a low content of heavy precursor may obviously yield small amounts of ³H labelled light DNA upon fragmentation (*Cleaver* 1969)

Incorporation of ³H-TdR into hybrid DNA of cells density labelled with BUdR prior to treatment (Scheme II in the present study) therefore seemed to be the method of choice for measuring DNA repair synthesis after exposure to mitomycin C To avoid incorporation of ³H TdR into hybrid DNA of control P-388 cells, the labelling scheme originally elaborated for HeLa cells (*Painter & Cleaver* 1967) had to be modified by reducing the initial period of growth in medium containing BUdR from 16 to 6 hours Using this modification only light DNA strands served as templates for DNA replication during labelling with ³H TdR Mitomycin C induced inhibition of DNA replication did therefore not affect the density of the DNA formed (Fig 3 left column) and this effect of the antibiotic did not contribute to the occurrence of ³H labelled hybrid DNA

Other mechanisms of mitomycin C action might theoretically contribute to the formation of ³H labelled hybrid DNA in this type of experiments Mitomycin C may cause extensive degradation of mammalian DNA (*Shatkin et al* 1962, *Kersten & Themann* 1962), and degradation of hybrid DNA might supply heavy precursor for semiconservative replication However, degradation of DNA in the P 388 cells occurs only after prolonged treatment with the antibiotic (*Ørstavik* 1972 b), rendering this explanation unlikely in the present study Furthermore, as mitomycin C acts as a difunctional alkylating agent (*Iyer & Szybalski* 1963, *Matsumoto & Lark* 1963), it might cause complex formation between ³H labelled light DNA and hybrid DNA Considering the low content of ³H labelled DNA of intermediary densities (Fig 3 f, g, h), it seems unlikely that this mechanism was of importance in the present experiments

The present demonstration of DNA repair synthesis indicates that the P-388 cells possess a mechanism for the repair of mitomycin C induced DNA lesions The existence of such a mechanism could account for the recovery of these cells after short treatment with the antibiotic It has previously been suggested (*Kersten & Kersten* 1969) that repair of mitomycin C lesions may follow the excision repair scheme originally proposed for repair of DNA in UV-irradiated bacteria (*Setlow & Carrier* 1964, *Boyce & Howard-Flanders* 1964) This would imply that reconstitution of functional DNA in mitomycin C treated cells is carried out by a process including an initial excision of alkyl groups from DNA, followed by repair synthesis as demonstrated in the present study, and finally a ligation of the reinserted bases to the intact part of the DNA molecule Investigations of other aspects involved in this hypothesis are in progress

Fig 3 Density profiles of DNA from P 388 cells grown in bromodeoxyuridine for 6 hours transferred to normal growth medium treated with mitomycin C and labelled with ³H thymidine (scheme II)

* Fractions selected for rebanding

Bandings in neutral CsCl carried out at 35 000 rev/min

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A RUBELLA HAEMAGGLUTINATION INHIBITOR SIMULATING ANTIBODY

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Pretreatment of sera with kaolin or with heparin $MnCl_2$ occasionally leaves non specific rubella haemagglutination inhibitors In doubtful cases flotation centrifugation through a NaBr solution with a density of 1 22 will distinguish inhibitor from antibody

Non specific rubella haemagglutination (HA) serum inhibitors are removed by treat ment with kaolin or heparin $MnCl_2$ (or dextran sulphate $CaCl_2$) before titrating HA inhibiting (HI) antibodies Heparin $MnCl_2$ is usually considered to be more specific in its action, as kaolin is claimed to remove some antibody as well The latter assumption is based on two observations a reduction of the total γ globulin content upon treatment with kaolin and the obtaining of lower HI titres after kaolin treatment than after other pre treatments In the first case it is presumed that the total antibody content reflects speci fic rubella antibody content, and, in the se cond case, that methods which yield higher HI titres do not leave non specific inhibitors

In earlier studies with antigen/antibody systems that are not influenced by inhibitors kaolin had no effect on the antibody titres against bacteria or viruses (5 6) It was found, however that mononucleosis and other heterophilic antibodies, as well as isoagglutinins and rheumatoid factor strongly adsorbed to kaolin This could not be explained solely by the dominating IgM in the latter group of antibodies, since cold agglutinins (anti I) and IgM antibodies to *Salmonella typhi* and rubella virus were not adsorbed to kaolin

The present report concerns a rubella HA inhibitor which closely resembles antibody The presence of such an inhibitor was suspected when we had the opportunity to ob serve a case of clinical rubella in an indivi dual who 4 months earlier (when she was 3 months pregnant) had a rubella HI titre of 20 in two serum samples (3, 4) Some properties of the inhibitor are presented, and methods to reveal false positive reactions are considered

MATERIALS AND METHODS

Sera The sera were selected from routine speci mens sent to the laboratory for rubella antibody titration The sera were examined fresh or after storage at −20° C

Rubella HI test Pretreatment with 12 5 per cent kaolin (Flow Laboratories Irvine, Scotland) was performed at pH of 8 5 for 20 min at room temperature with repeated shakings Pretreatment with heparin $MnCl_2$ was performed according to *Cooper et al* (1) The HI titration was carried out essentially as described by *Stewart et al* (9)

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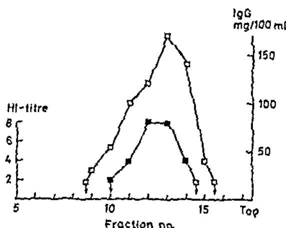


Fig 1 Sucrose gradient centrifugation of a heparin $MnCl_2$ treated serum showing a false positive rubella HI reaction The HI active material cannot be separated from IgG ■——■ HI titre □——□ IgG

Ultracentrifugation Sucrose (12 5 37 5 per cent w/v) gradient velocity centrifugation was performed with the SW 39 rotor in the Beckman Spinco Model 50L ultracentrifuge at 100 000 × *g* for 18 h 0 2 ml of serum was layered over 4 8 ml of the gradient, and after centrifugation fractions of about 0 3 ml were collected by puncturing the bottom of the tubes

Flotation centrifugation was performed at 100 000 × *g* for 16 h after layering 4 7 ml of a NaBr solution with a density of 1 22 over 0 1 ml of the serum adjusted to a density of 1 30 by addition of NaBr

Hydroxyl apatite chromatography was performed with a 10 × 40 mm hydroxyl apatite (Bio-Gel HTP, Bio Rad Labs Richmond Calif) column equilibrated and run by step wise elution as described by *Hjertén* (7) 0 2 to 0 4 ml of serum was applied to the column

Absorption of sera with Staphylococcus aureus *strain Cowan 1* The bacteria were grown on nutrient agar for 18 h Thereafter the agar surface was scraped and the harvested bacteria were washed twice in s[illegible] Equal volumes of packed bacteria and serum [illegible] fraction) were mixed and the bacteria [illegible] down after incubation for 1 h at [illegible]

Inhibition of IgG [illegible] *fied protein A* Purified protein A w[illegible] Dr *A Grov* in this department The [illegible] performed as described by *Grov* (12 with [illegible] of protein A and IgG corresponding to the [illegible]alence zone of precipitation

Quantitation of IgG This w[illegible] rmed by single radial diffusion against an[illegible] Partigen IgG Behringwerke AG)

EXPERIMENTS AND RESULTS

As mentioned in the introduction the present studies were prompted by the observation of a characteristic clinical rubella in an individual (T H) who, on the basis of prior serological examinations was considered to have experienced a rubella infection earlier The HI activity demonstrated in the earlier sera mimicked HI antibody activity in several respects The properties of the rubella HI active material in these sera will therefore be described in detail

1 An HI titre of 20 was reproducibly obtained with kaolin treated sera The titre was 2 to 4 times higher when serum was pre treated with heparin MnCl

2 Sucrose gradient centrifugation was carried out with heparin MnCl treated serum Each fraction was tested for HI activity and IgG content (Fig 1) It is seen that the HI activity was recovered in the IgG containing fraction It is especially noted that no HI activity was demonstrated in the top fractions containing the β lipoproteins which are considered to represent the non specific inhibitors of rubella HA

3 Absorption of HI activity by *Staphylococcus aureus* Cowan 1 This bacterium is rich in protein A which specifically binds most of the serum IgG The HI peak fraction from sucrose gradient centrifugation was treated with the bacteria All HI activity was removed

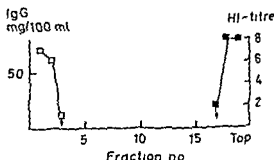


Fig 2 Flotation centrifugation through NaBr d = 1 22 of the same serum as shown in Fig 1 Inhibitors are now separated from IgG ■——■ HI titre □——□ IgG

The results of 1, 2 and 3 are compatible with the HI active material having the character of an antibody Since, however, this individual a few months later got a typical, clinical rubella with specific IgM rubella antibody, and since there was a striking difference in titre between sera treated with kaolin and heparin $MnCl_2$, the presence of a non specific HA inhibitor was still suspected The HI activity was therefore studied further by other methods

4 The HI peak fraction from sucrose gradient centrifugation was treated with purified protein A as described under Methods The HI activity remained unchanged after this treatment A control experiment was therefore performed with a rubella HI antibody negative serum, which was treated with *Staph aureus* Cowan I It appeared that most of the HI inhibitory activity was removed by the staphylococci This strain accordingly removes lipoproteins as well as IgG

5 Hydroxyl apatite chromatography The serum was applied to the column and stepwise elution was performed

No HI active material appeared after elution with 0 1 and 0 25 M phosphate which should elute antibody After elution with 0 65 M phosphate, however some HI active material was obtained This showed that on this column the HI active material behaved like a lipoprotein (6)

6 Flotation centrifugation The result of a flotation centrifugation is shown in Fig 2 It is seen that the HI active material had a density which was lower than 1 22 since all activity was found in the top fractions while the IgG remained at the bottom The HI active material is accordingly a lipoprotein This experiment provides the conclusive evidence for the non specific nature of the rubella HI active material of this serum

Re Examination of Other Sera with Low HI Titre

Rubella HI titres of 10 to 0 are not often obtained with sera from young people As a consequence of the above-mentioned findings, we re examined 23 sera with a titre of 10 or 20 obtained last year from student nurses The sera were examined both after treatment with kaolin and heparin $MnCl_2$ This time two of the sera showed a titre below 10, while one serum was studied further because the titre after heparin-$MnCl_2$ treatment was 160 as compared to 20 after kaolin treatment Flotation centrifugation (as illustrated in Figure 2) showed that all inhibitory activity of this serum was caused by a lipoprotein When a new serum sample was taken half a year later, this inhibitor was not present, the titres being <10 and <8 after pretreatment with kaolin and heparin $MnCl_2$, respectively It ought to be mentioned that at the time of the first serum sample this individual was pregnant in the first trimester

The Rubella Haemagglutinin as an Antigen in the Complement Fixation Test

The HA antigen with an HA titre of 256, was examined by checker board titration against positive sera in the CF test An antigen dilution of 1 4 was found to be optimal Twenty four sera, 6 negative and the rest having HI titres from 20 to 320, were examined from dilution 1 2 5 One serum with an HI titre of 20 was doubtfully positive, the other 17 HI positive sera were positive also in this CF test The ratio HI/CF titre was 8 1 with 13 sera 16 1 with 2 sera 4 1 with one serum

The sera were also examined by this CF test after treatment with kaolin The titres remained unchanged

Higher titres were obtained by *Vesikari et al* (10) using purified virus as a CF antigen Our antigen is most probably predominantly made up of fragments of the viral envelope Only IgG reacted in the test

The CF titre using the HA antigen showed no parallel to the CF titre obtained with the traditional rubella CF antigen (cell-associated)

Prolonged Treatment with Kaolin

Serum from our patient (T H) was treated with different concentrations of kaolin, at different temperatures and times When the amount of kaolin was increased, no more inhibitors were removed Decreased amounts of kaolin removed less inhibitors The only way to obtain complete removal (*i e* titre below 10) of inhibitors from this serum was to prolong the treatment up to at least two hours at room temperature or at 4° C

DISCUSSION AND CONCLUSIONS

The occasional occurrence of false positive rubella HI reactions has been reported by several investigators This has lead to a rather sceptical attitude towards the evaluation of low HI titres Thus titres of 10 and 20 are not uncommonly excluded in epidemiological studies When faced with the question of whether a pregnant woman may contract a rubella infection, we have to provide an answer as to her immune status Beyond doubt some individuals have rubella HI antibody titres at the 10 or 20 level and are immune against a primary infection Therefore, in the routine laboratory we have to give the clinician a definite answer as to the immune status of the individual concerned

From earlier (6) and present screening of sera it is clear that both kaolin and heparin $MnCl_2$ efficiently remove non specific inhibitors from almost all sera An occasionally occurring inhibitor which was particularly resistant to the pretreatments appeared to belong to the lipoproteins but our limited material does not allow general conclusions The false positive sera presented here showed a strikingly higher titre after treatment with heparin $MnCl_2$ than with kaolin, and we are inclined to consider the heparin $MnCl_2$ method less suited than the kaolin method We have earlier recommended chromatography on a hydroxyl apatite column or sucrose gradient centrifugation for distinction of inhibitors from antibodies The latter method was here shown to fail because the density of the top fraction was too low On the basis of our present findings we will briefly summarize some precautions to be taken when evaluating low rubella HI titres and suggest possible supplementary examinations

1 Treatment with kaolin should be prolonged for up to at least two hours Kaolin does not remove specific antibody

2 Any rubella HI titre of 10 and 20 should be looked upon with suspicion

3 In suspected cases the titres after treatment with kaolin and heparin $MnCl_2$ should be compared If the titre is higher after treatment with heparin $MnCl_2$, an HI inhibitor is likely to be involved

4 A potent HA antigen used in a CF test will give a titre which is 1/8 of the HI titre, provided the antibody is of the IgG class A positive CF test indicates immunity

5 Flotation centrifugation through a NaBr solution with a density of 1 22 will provide the final answer as to the nature of the HI active material

6 Checking for residual inhibitors against the HA of Sindbis virus, which is highly sensitive to β lipoprotein inhibitors (8), is a method which might prove useful but has not been attempted in our cases

In conclusion Until reliable methods are available for removal of all non specific rubella HI active material low level immunity against a primary rubella infection should not be diagnosed until verified by flotation centrifugation

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REGIONAL LYMPH NODE RESPONSE IN RATS TO INJECTION OF SEMI-ALLOGENEIC LYMPHOCYTES

Time, Weight, Dose, and Histocompatibility Relations

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Subsequent to injection of F_1 hybrid lymphoid cells into the hind footpads of the parental strain rats the weight of the popliteal lymph nodes increase with a maximum gain 4 to 6 days after the injection The enlarged lymph nodes obtain weights approximately 4 5 times greater than normal When lymphocytes in doses between 2 × 10^6 and 20 × 10^6 per footpad are injected, the node weights increase with a slight dose dependency, but weights never reach the magnitude as in local lymph node G V H assays Cells from different lymphoid organs had varying antigenic effect, blood-, lymph node , and spleen lymphocytes were the best stimulators, whereas bone marrow and thymus were less effective The weights were found to vary depending on the rat strain used as responder When closely related rat strains were employed as donor recipients the weight maximum was found on the same days and was of the same magnitude as in widely disparate strain combinations F2 hybrid rats responding against major histocompatible injected F1 hybrid lymphocytes did not produce a weight increase at day 5, but the nodes were found enlarged one week later in the postinjection period

The popliteal lymph nodes in F1 hybrid rats enlarge and gain considerably in weight following injections of parental strain lymphocytes into the footpads, and the weight gain reflects the graft-versus-host (GVH) activity of the injected cells against the F1 hybrid (3)

The present report gives a description of the growth characteristics of the draining lymph node in parental strain rats injected with allogeneic F1 hybrid lymphoid cells These investigations have been undertaken to clarify whether the weight response is correlated with the host-versus graft (HVG) activity, an activity dependent on the immunogenetic disparity between recipient and donor

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MATERIAL AND METHODS

Inbred rats of the Fischer (Fi), Lewis (Le), AS Wistar (Wi), and Brown Norway (BN) strains and their F1 hybrids were used The strains are derived from the stock at the Institute for Experimental Immunology and the Fibiger Laboratory in Copenhagen

The Fi, Le and AS strains are generally considered to be identical at the major rat histocompatibility locus (Ag B locus) (8), and carry the AgB1 allele

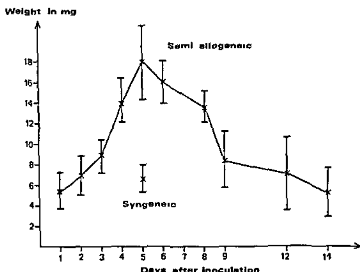


Fig 1 Typical growth curve Donor F1/W1 F_1 hybrids 9 × 10^7 lymph node cells injected per foot pad Recipient F1 parental strain rats 2 months old The maximum weight gain is on day 4–6 after immunization The vertical lines indicate S D

The W1 and BN respectively, carry the AgB2 and AgB3 alleles and thus d ffer from each other and from the above with respect to strong trans plantation antigens

The rats were aged from 2–6 months when used

The lymphoid tissue used as cell source for in jections was removed aseptically from the animals under aether anaesthes a The tissues were teased into small pieces filtered through a double gauze layer and washed twice Washings were performed in Tissue Culture Medium 199 (TC 199) with Earles salts and 20 mM Hepes (No 1 048 B Flow Lab) The number of lymphoid cells were counted in a Burker Turk chamber and the percentage of dead cells determined by trypan blue uptake The viability of the lymphoid cells used for injection always exceeded 90 per cent

Lymphocyte suspensions in TC 199 were injected subcutaneously in 0 15 0 25 ml volumes into the loose tissue in the hind footpads of the recipient rats After varying intervals the rats were killed by aether and the popl teal lymph nodes were re moved freed from fat and weighed to the nearest 0 5 mg

The parameters for the popliteal lymph node we ght response were determined for groups of rats of the same age and sex injected on the same day with cell suspensions from a common pool of cells

The data to be presented in the following are based upon experiments using a total number of rat recipients of approximately 400

W lcoxon s rank sum test was used for statistical calculations

RESULTS

Time weight Relationship

Enlargement and weight gain of the pop liteal lymph nodes was observed in parental strain rats injected with a standard dose of 0 9 × 10^7 F1 hybrid spleen lymphocytes per footpad The increase in weight in the major incompatible strain combinations W1 × F1 → W1, W1 × BN → W1, F1 × BN → F1, have shown a uniform time relationship in all combinations The maximum weights were found on days 5–6 after injection, and return to normal weights was observed after day 10–12 The typical growth curve is presented in Fig 1, and as control for non immunolo gically induced growth stimulation the lymph node weights of rats injected with syngeneic spleen lymphocytes are also shown in the figure

Different Lymphoid Cell Populations Used as Antigen

F1 hybrid lymphoid cells from thymus, bone marrow, spleen, lymph nodes, and pe ripheral blood as well as red blood cells have been tested in the W1 × F1 → W1, and the

TABLE 1 *The Immunogenecity of Different Lymphoid Cells The Lymph Node Weights after Injection of Different Kinds of Cells The Weights Are Determined to the Nearest 0 5 mg, and the Presented Values Are the Arithmetic mean ± S D Each Group Represents at Least 8 Lymph Nodes*

Cell inocula from Wi/F1	Recipient	
	W1	F1
Lymph node cells*	22 6±1 7	12 8±1 5
Bone marrow cells	18 1±1 8	8 4±1 9
Spleen cells	23 4±1 2	14 4±1 7
Blood lymphoid cells		15 8±1 6
Red blood cells§	7 4±0 6	5 4±1 7
Thymic cells	18 1±2 1	10 4±1 2
Syngeneic cells		
Lymph node cells	6 8±1 4	4 6±1 0

* The doses of lymphoid cells injected are 2×10^7
§ Red blood cells are injected in doses of 2×10^9
The cells used as inocula are in each recipient group from the same Wi/F1 donor

F1 × W1 → F1 combinations for their immunogeneic potency in the local lymph node assay It was found that injections of equal numbers of lymph node , spleen , and blood lymphocytes gave rise to weights of the same magnitude, weights that were approximately 20 per cent greater than those obtained using thymus or bone marrow lymphocytes in the same dose (Table 1) Erythrocytes were found to be very poor stimulators in this system, giving non significant responses ($p > 0.05$ Wilcoxon rank sum test) even in doses 100 times greater than the corresponding lymphocyte doses

Antigen Dose weight Relations

The number of spleen and lymph node lymphocytes injected per footpad has been varied in a group of experiments from 2×10 to 2×10^7 viable cells The relation between the number of cells injected and the maximum weight obtained (day 5 response) was determined in 5 different major incompatible donor recipient combinations and the results shown in Fig 2 are typical Doses of 3×10^6 and above caused significant ($p < 0.05$) increase in lymph node weight in 4 out of 5 combinations Cell numbers between 3×10^6

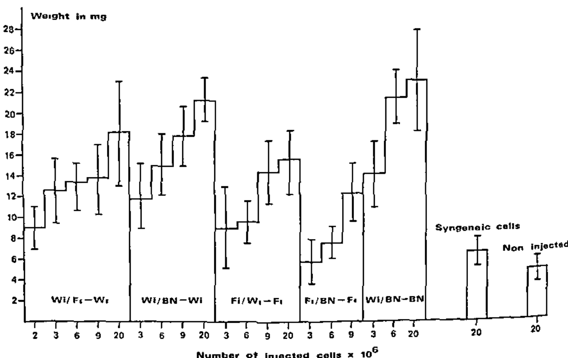


Fig 2 Dose response curve for the combinations dealt with in the text Donor cells were F_1 hybrid lymph node cells in the doses indicated Parental strain rats were recipients All lymph nodes were harvested on day 5 The horizontal lines represent the arithmetic mean and the vertical lines the S D

TABLE 2 *Immunogenetic Disparity Measured by GVH The Relative Antigenic Gap between the Strains Used Was Measured in GVH Assays by Injecting Parental Strain Lymph Node Cells in Doses of 9 × 10⁶ into the Foot Pads of the Corresponding F_1 Hybrid Rats (Aged 6–8 weeks) The Weights of the Popliteal Lymph Nodes Were Determined on Day 7*

Combination	W1→F1/W1	W1→W1/BN	F1→F1/W1	F1→F1/BN	BN→W1/BN	BN→F1/BN
Lymph node in mg ± S.D	41 0±15 9	45 0±11 0	16 5±2 2	26 5±6 2	36 0±5 6	34 0±7 2

The W1 results are pooled results, whereas the other observations are from single experiments

and 9 × 10⁶ provoked intermediate reactions, and doses of 9 × 10⁶ and up to 2 × 10⁷ gave responses 2–4 times greater than syngeneically injected cells

Weight Responses in Different Histo incompatible Combinations

The weights of lymph nodes draining injected footpads were found the lowest if F1 rats were used as *recipients* This held true for all allo antigens tested and in all doses BN on the contrary responded with the heaviest lymph nodes against the W1 antigen whereas BN against F1 (not illustrated) responded in the same order of magnitude as W1 against BN and W1 against F1 The relationship between weight and donor recipient combination does not correspond fully with the "strength' of the histo-incompatibilities between these strains as determined in *GVH assays* (Table 2)

HVG Against "Weak' Histocompatibility Antigens

Injection experiments using AgB identical minor disparate strains i e Le cells injected *in F1 recipients, and F1 cells in AS recipients*, have been performed These experiments have only been performed using parental lymphocytes as antigen Fig 3 shows the results A "normal day 5–6 maximum and a 'normal" weight decrease thereafter was consistently found The weight assay did not show any difference in the former strong and

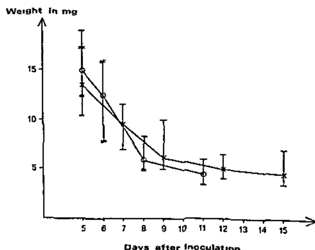


Fig 3 The time/weight relationship of the popliteal lymph nodes in Le rats after injection of 2 × 10⁷ F1 lymph node cells (x) and in AS rats after injection of 2 × 10⁷ F1 lymph node cells (O) The crosses represent the median values of at least 8 lymph nodes and the vertical bars the range

TABLE 3 *HVG Lymph Node Reactions against Minor Antigens Lymph Node Weights in F_2 Hybrids Selected Serologically to Carry the AgB 1 (F_1) and AgB 3 (BN) Genotype F_1/BN F1 Hybrid Lymph Node Cells Were Injected as Antigen in a Dose of 9×10^6 Viable Cells Per Foot Pad Right and Left Lymph Nodes Were Harvested on Day 5 and 11*

Rat No	Harvest day 5	Harvest day 11
1	5 5 mg	14 0 mg
2	8 5 mg	13 5 mg
3	6 5 mg	7 0 mg
4	10 0 mg	12 5 mg
5	7 0 mg	6 0 mg
6	10 0 mg	12 5 mg
7	5 0 mg	5 5 mg
8	11 0 mg	16 0 mg

these latter "weak" histocompatibility barriers

F2 hybrid rats between the F1 and BN strains were raised and the AgB type of the individual rats was determined serologically using anti F1 and anti BN immune sera in lymphocytotoxicity and haemagglutination techniques Local lymph node assays were performed on these F2 hybrids injected with parental strain or F1 hybrid spleen cells carrying their own AgB type The weight and growth reactions in the popliteal nodes which are bound to be caused by varying numbers of non AgB antigens (minor) are shown in Table 3 Five out of eight of these rats had high lymph node weights late, (day 11), higher than weights on day 5, and the day 11 weight was of the same order of magnitude as the day 5–6 response in strong combinations These results have shown the kinetics of the HVG response in the local lymph node against certain non AgB antigens to be quite distinct from the response in strong combinations

When F2 rats were injected with major incompatible lymphocytes the normal day 5 maximum was found (not illustrated)

The Experimental Variation

The S D in the pooled resul s as shown in Fig 2, with no less than 20 observations in each dose group, varies from 2 2 5 5, and the S D of the mean in the same results are from 0 5–2 0 The S D in a single experiment, as illustrated in Table 1 (F1 response) varies from 1 0–1 9, and the S D of the mean for these results are 0 6–0 9

DISCUSSION

Several authors have described a weight gain in lymph nodes after antigen challenge within the area drained by the actual lymph node *Scothorne* (10) after skin grafting in rabbits *McKhann* (5) after skin grafting in mice, *Ostrowski et al* (7) after inoculation of irradiated tumour cells in mice, and *Taub & Gershon* (13) after injection of adjuvant materials also in mice

Scothorne's experiments gave evidence that the weight gain was "specific" for the draining ipsilateral lymph node, as no weight gain was observed in the contralateral node or in the spleen In a later paper (9) by the same author the histological changes in the draining nodes were described, the main finding being large blastoid cells appearing in the lymph node as early as 72 hours after grafting These cells were derived from precursors among the reticular cells in the draining lymph node and could be detected within 24 hours after antigen challenge (11)

Taub found that synthesis of DNA in the regional lymph node expressing the "in loco cell synthesis, peaked 5 days after injection of adjuvant material, coinciding with the maximum weight gain, but not related to the ascending phase of the weight increase In this way it was evidenced that the observed enlargement to a great extent represented the result of an influx of cells from the circulating pool of lymphocytes This local lymphocyte trapping was also described by *Zats & Lance* (16) in a mouse system after challenge with heterologous antigens (sheep red blood cells, agglutinable Salmonella typhi H antigen or Keyhole limpet haemocyanin), and also after allogeneic skin grafting

After grafting, a marked lymphocyte trapping in the regional node was found, ex

pressed as the ratio between the accumulation of injeted ^{51}Cr labelled cells in the regional node and the contralateral node The ratio exceeded 1 0 after 24 hours and reached a maximum of 1 8 after 9 days in relation to first set skin allograft rejections In second set rejections the ratio obtained 24 hours after grafting was 1 4, with the maximum trapping (ratio 1 7) going on 2 days after grafting The argument that the trapping during the early phase of the lymphocyte accumulation was a non specific event has been put forward (2 16) This is consistent with the results in the heterologous system in that the antigen sensitive cells to these antigens represent only approximately 10^{-5} of the lymphocytes circulating in the blood (4)

In allograft systems as well as in the system dealt with in this paper there are approximately 2 per cent lymphocytes in the blood that react against the histocompatibility antigens in question (15) It is therefore more plausible that the weight increase may to some extent be the result of a specific lymphocyte trapping when this figure is taken into account This possibility will be dealt with in a later paper

As seen in Fig 2 F_1 rats showed a smaller lymph node response than the other rats despite the fact that these rats differ at the AgB locus from the different stimulating donor strain cells A reasonable explanation of this controversy was given by *Vikliky et al* (14) in a report describing allo-cluster forming cells in the regional lymph node after antigeneic stimulation suggesting that in certain mice there is an upper limit for the immunological proliferation a limit determined by the genotype of the animal itself

McKhann (5) and *Sparck* (12) found that the smaller the immunological disparity between donor and recipient in HVG assays in mice the greater would be the maximum weight gain and the max mum gain appeared later following an g n challenge in weak combinations

This finding c u d not be reproduced n the present study ng Le F_1 and As ts in the donor hos umb nations These rats are generally believed to be identical at the strong histocompatibility loci and the immunogenetic differences should therefore only be minor ones The experiments on these non AgB different combinations, however, were only done in parental to parental combinations with the possibility of a combined GVH HVG reaction Similar parental to parental reactions in strong combinations have been carried out in later experiments (unpublished) and have shown no greater lymph node weight at day 5–6 in these two way reactions compared with pure HVG reactions (F_1 to parental)

When the AgB typed F_2 hybrids and the AgB identical F_1 hybrids were used in recipient donor combinations (again possibility for two way reaction) it was however, demonstrated that a less pronounced weight increase was found on day 5 and a maximum later in full agreement with the above mouse data

This finding taken together with other observations (1 6) may lead to a questioning of the serologically determined AgB identity of Le F_1 and As rats employed

The present paper has primarily dealt with the regional lymph node *weight* as parameter for the HVG response Further work concerning this system is being done in this laboratory and later reports will deal with 1) The MLC response and G V H response using regional lymph node lymphocytes as responder cells 2) The regional lymph node weight and M L C responses in pre immunized recipients 3) H V G G V H interactions in parental to parental combinat ons 4) Production of antibodies lympho-kines and effector cells in the regional lymph node in relation to H V G

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IN VIVO REACTIONS OF STAPHYLOCOCCAL ANTIGENS

I Hypersensitivity to Protein A

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Results are presented to show that protein A from *Staphylococcus aureus* elicits delayed hypersensitivity reactions in sensitized animals as well as early reactions mediated by immunoglobulins in normal and sensitized animals Delayed hypersensitivity to protein A can be induced in guinea pigs and rabbits by a single injection of 50 μg protein A in complete Freund s adjuvant and is detected after 10 days It can also be transferred from sensitive to normal animals by lymphoid cells but not by immune sera

Protein A from *Staphylococcus aureus* possesses the ability to elicit hypersensitivity reaction of the immediate type in non immunized animals Normal rabbits develop passive Arthus reaction after injection of protein A subsequent to intravenous administration of human IgG (8) It is also possible to evoke a local passive Arthus phenomenon after intradermal injection of preformed aggregates of protein A and normal human IgG (8) *Gustafson et al* (9) have reported local anaphylaxis in non immunized guinea pigs after intradermal administration of even trace amounts of protein A Higher doses of the antigen given intracardially caused systemic reactions Anaphylaxis produced in guinea pigs by protein A can be blocked by antihistamine drugs (9) Protein A has also been shown to provoke the wheal and erythema reaction in man this reaction being the human counterpart to cutaneous anaphylaxis (12)

Hypersensitivity reactions of the immediate type are the result of a reaction between protein A and the Fc part of normal IgG IgG with ability to interact with protein A through Fc has been demonstrated in sera from guinea pigs mice and men (2, 3, 4) and in trace amounts in rabbit sera (5, 7)

Taubler (15) and *Taubler & Mudd* (16) have successfully demonstrated delayed hypersensitivity in mice to crude staphylococcal extracts induced by viable cocci Similar reactions have later been reported to some constituents of staphylococcal cell walls teichoic acid, mucopeptide and teichoic acid mucopeptide complexes (11) However, delayed type allergy to protein A has not been reported

This study was undertaken to reexamine the action of protein A on the skin of normal animals and to search for delayed reactions in animals sensitized by various routes to protein A

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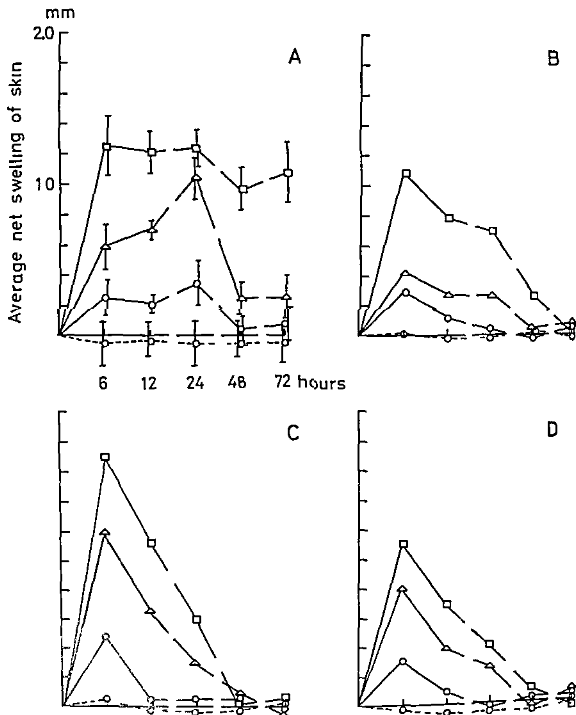


Fig 1 Skin reactions n sensitized guinea pigs after testing with protein A
A Animals sensitized with 100 μg of protein A in FCA,
B Animals sensitized with 100 μg i protein A in FICA
C Animals sensitized with 100 μg of protein A in SS
D Control animals
—○— protein A 10 μg, —△— protein A 50 μg,
—□— protein A, 100 μg, - -○- saline

MATERIALS AND METHODS

Antigens

Crude protein A was isolated from *S aureus* strain Cowan I according to the procedure described previously (6)

Experimental Animals

Outbred albino guinea pigs weighing 350–400 g living in a closed colony, and adult albino rabbits, weighing 3–4 kg, of both sexes, were used throughout

Sensitization

Groups consisting of 10 guinea pigs or 5 rabbits were immunized with

A A saline solution of protein A (100 μg) emulsified in an equal volume of complete Freund s adjuvant (FCA) (Difco)

B 100 μg of protein A in saline mixed with an equal volume of incomplete Freund s adjuvant (FICA) (Difco), and

C 100 μg of protein A in isotonic saline (SS) The immunizing volume of 0 5 ml was distributed equally into both hind footpads

Booster sensitization given to animals 14 days later was an exact repetition of the first injection and was introduced into both fore footpads

Skin Tests

Ten days after sensitization guinea pigs and rabbits were tested intradermally in the depilated flanks with 0 1 ml volumes containing 10 50 and 100 μg of protein A in isotonic saline Skin thickness was measured just before and 6, 12, 24 48 and 72 h after injection using a precise caliper Three readings were made on each site of injection and the average net swelling of skin with standard deviations was calculated for each group of animals

Histology

Reaction sites were removed for histology fixed in 4 per cent buffered formalin embedded in paraffin and sectioned at 5 μ The sections were stained with hematoxylin and eosin or Giemsa stain

Preparation of Cell Suspension

On the tenth day after sensitization the donor guinea pigs and rabbits were bled out by cardiac puncture Their popliteal inguinal and mesenteric lymph nodes and spleens were removed and trimmed free of fat, then bathed in Hanks solution supplemented with 10 per cent of homologous heat inactivated serum The procedures for preparing cell suspensions counting the number of leukocytes and determining percentage viability of cells were derived from *Harris et al* (10) Finally the leukocyte concentration was adjusted to 5×10^8 to 1×10^9 cells per ml

Transfer of Cells

The cell suspensions were well mixed and injected intraperitoneally into recipient guinea pigs or intravenously into rabbits The recipient animals were tested intradermally with 50 μg of protein A in 0 1 ml volume immediately after the transfer and then daily for 9 days Readings were made 24 h after the injections

RESULTS

Protein A gave positive skin reactions in sensitized guinea pigs increasing with the dose of antigen (Fig 1) In animals sensitized with protein A mixed with FCA a delayed reaction was clearly visible 24 h after testing Large amounts of antigen (100 μg) used for testing provoked a strong early inflammation of skin masking the delayed hypersensitivity Sensitization of guinea pigs with protein A emulsified in FICA or given in saline resulted only in an Arthus type hypersensitivity, this reaction being maximal after 6 h Control animals also revealed early inflammation and the intensity of skin oedema was nearly the same as in animals sensitized with protein A in FICA

Booster sensitization with 100 μg of protein A in FCA and/or in FICA distinguished clearly between early and delayed reactions (Fig 2) Traces of delayed reactions were also found in guinea pigs resensitized with protein A in SS and even in the control animals This group was, in fact, sensitized intracutaneously during the first testing

Very strong reactions were found in sensitized rabbits (Fig 3)

Protein A mixed with FCA or FICA evoked both early and delayed reactions whereas rabbits sensitized with protein A in SS appeared to be practically unaffected Skin reactions to protein A in the latter group of animals (Fig 3C) were discrete and comparable to the traces of inflammation found in control rabbits

After the second sensitization of rabbits (Fig 4) the early inflammation remained

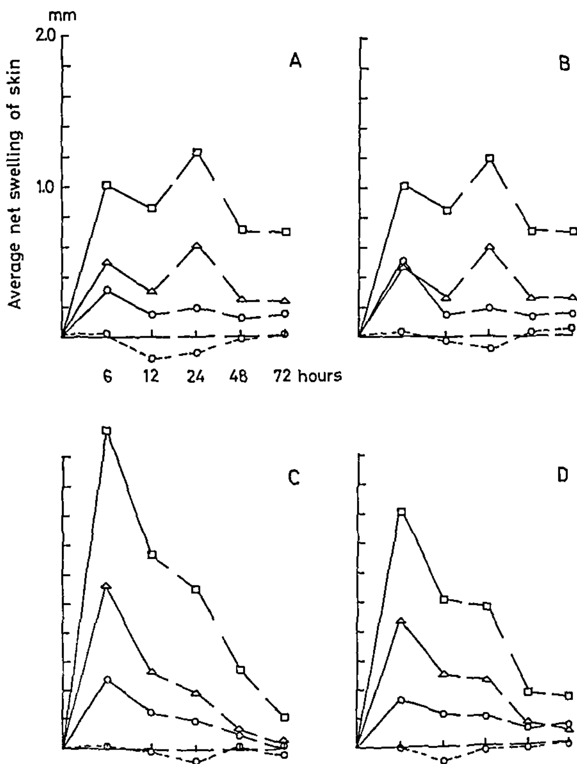


Fig 2 Skin reactions in resensitized guinea pigs after testing with protein A
A Animals sensitized with 2 × 100 μg of protein A in FCA,
B Animals sensitized with 2 × 100 μg of protein A in FICA,
C Animals sensitized with 2 × 100 μg of protein A in SS,
D Control animals Symbols see Fig 1

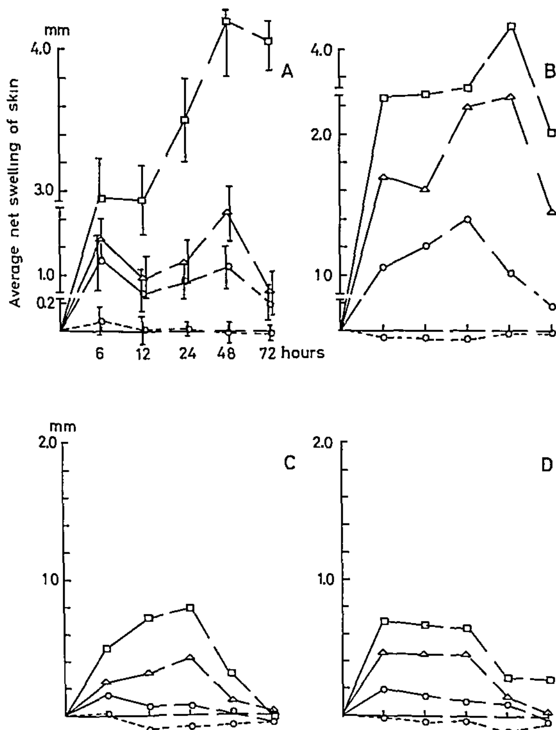
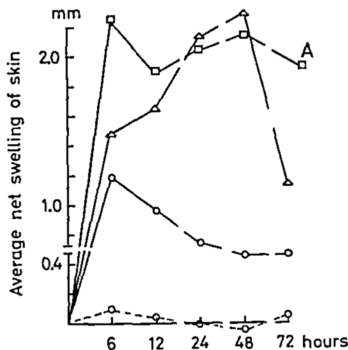
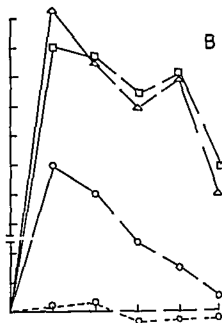
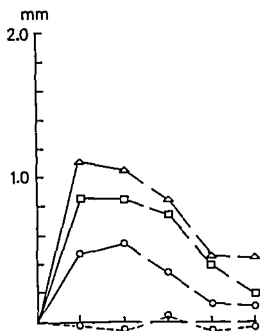


Fig 3 Skin reactions in sensitized rabbits after testing with protein A See footnotes for Fig 1







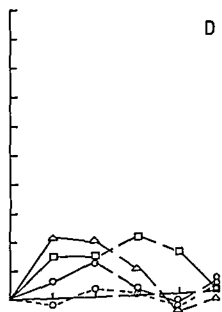


Fig 4 Skin reactions in resensitized rabbits after testing with protein A See footnotes for Fig 2

nearly constant but in both groups (A and B) the intensity of the delayed reaction diminished

Microscopic examinations of 6 h reaction sites taken from both sensitized and normal guinea pigs which had been tested intradermally with 50 μg of protein A showed extensive polymorphonuclear exudate of epidermis dermis and muscle layer (Fig 5) Polymorphonuclear cell infiltration was persistent throughout the course of inflammation in the group of control animals In guinea pigs sensitized with protein A in FCA infiltration of the skin was after 24 h displaced to the deep dermis and mononuclear cells came to dominate the picture (Fig 6) Animals sensitized with protein A in FICA or in SS showed after 24 h an abundant infiltration of the skin composed mainly of polymorphonuclear leukocytes and of single mononuclear cells



Fig 6 Guinea pig skin 24 h after intradermal injection of 50 μg protein A Animal sensitized with 100 μg of protein A in FCA (Hematoxylin eosin × 150) Mononuclear cell infiltration is seen in the deep dermis and in the underlying fatty tissue

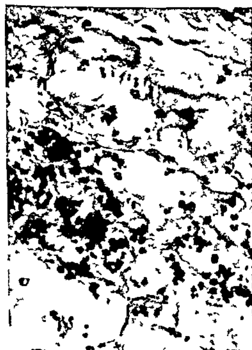


Fig 5 Guinea pig skin 6 h after intradermal injection of 50 μg protein A Animal sensitized with 100 μg of protein A in FCA (Hematoxylin eosin × 150) An extensive infiltration of polymorphonuclear cells is seen in the border region between the dermis and the subdermal fatty tissue

Very similar pictures of the 6 h early inflammation were observed in the skin of rabbits sensitized with protein A in FCA and FICA The latter group showed very intensive deep infiltration After 48 h the exudate showed an agglomeration of almost exclusively mononuclear cells mainly lymphocytes Only a trace of inflammatory reaction with discrete polymorphonuclear infiltration was found in control rabbits

The findings above strongly suggesting the development of delayed hypersensitivity to protein A in sensitized animals were confirmed by passive transfer of lymph node and/or spleen cells taken from sensitized donors

Analysis of Giemsa stained smears of spleen cell suspensions revealed about 75 per cent lymphocytes and 15 per cent polymorpho

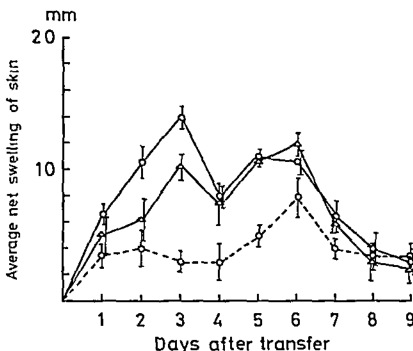


Fig 7 Skin reactions in recipient guinea pigs after passive transfer of sensitized cells and testing with 50 μg of protein A —○— lymph node cells recipient animals —△— spleen cells recipient animals - -○- - control animals

nuclear leukocytes The rest was composed of monocytic and basophilic leukocytes and blast cells Lymph node cell suspensions appeared more homogeneous consisting of about 95 per cent lymphocytes and trace numbers of other cells

Cell viability was checked by the dye exclusion technique, which revealed that 20–35 per cent spleen cells and 10–20 per cent lymph node cells did not take up the trypan blue stain

Recipient guinea pigs were most sensitive on the third day after intraperitoneal transfer and the animals apparently retained passive sensitivity for 6 days (Fig 7) However from the fourth day after the transfer a general increase of the skin reactions occurred most probably due to superimposed active sensitization by the test doses

Passive intravenous transfer of sensitized spleen cells into recipient rabbits excited violent skin reactions after 48 h (Fig 8)

These reactions quickly diminished in the next 2 days Control intravenous injections of 5 ml of immune serum against protein A (titre of the precipitins, as tested in double diffusion in agar was 1/128) showed a passive Arthus reaction after 24 h which disappeared after 48 h

DISCUSSION

On the basis of the gross appearance of the inflammatory lesions and their time dependence (1) and of the histological picture (14) it was possible to demonstrate delayed type hypersensitivity to protein A in specifically sensitized animals This was confirmed by the successful passive transfer of the hypersensitive state into normal animals by viable cells but not by antibodies

Both normal and sensitized guinea pigs revealed the well known early Arthus type reaction as the result of a pseudoimmune reaction (2) *Taubler* (15, 16) has also demonstrated the immediate reaction to extract of disrupted staphylococcal cells in sensitive and normal mice masking the delayed allergy This reaction was probably evoked by the protein A content in his preparation Re

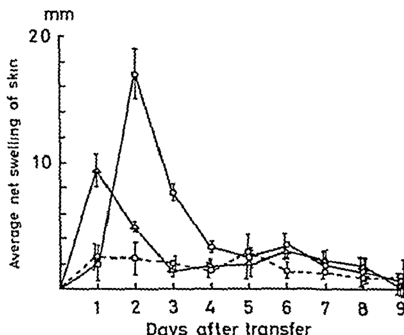


Fig 8 Skin reactions in recipient rabbits after passive transfer of sensitized cells or immune serum and testing with 50 µg of protein A ——○—— spleen cells recipient animals, ——△—— immune serum recipient animals – –○– – control animals.

peated sensitization or use of large amounts of the antigen enhanced early reactions due to stimulation of antibody production Apparently, the humoral response to protein A in guinea pigs does not suppress the delayed cellular sensitivity

The early reactions in normal unsensitized rabbits and in the animals sensitized with protein A in SS were very slight in accordance with the weak reaction observed between normal rabbit IgG and protein A (5 7) The use of adjuvant or resensitization strongly promotes Arthus type sensitization as a result of the aggregation (in dermis) of Fab reacting (13) immune rabbit IgG by protein A Obviously an excess of the antibodies after resensitization reduced the delayed hypersensitivity in our studies (18)

Experiments with passive transfer of cells showed that guinea pigs are very sensitive to protein A and even intradermal te t ng with small amounts of the antigen evident y results in active sensitization This sensitization is certainly of humoral type the phenomenon not having been observed in rabbits For this reason the termination of the passive sensitivity to protein A in guinea pigs is impossible to determine As positive reactions in rabbits disappear within 4 days after transfer, our animals probably have a low degree of genetic identity (17), but the occurrence of the hypersensitivity reaction after passive transfer into rabbits is not fully understood

It is of interest that contrary to the calculations of *Turk* (18), better transfers were obtained with lymph node cells than with spleen cells in spite of the number of transferred cells being identical Probably lymph nodes, as a regional lymphatic organ draining the site of injection, contained a higher number of sensitive cells than the spleen, the time since antigenic stimulation being so short (10 days) Moreover the percentage of lymphocytes in lymph node cell suspension was higher

Previous studies (2, 3, 4, 5 8, 9, 13) and the experiments shown here clearly indicate that protein A possesses more antigenic de

terminants capable of reacting in different ways unspecifically with normal IgG, specifically with immune IgG and additionally with sensitized cells

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ANTIBODIES AGAINST STAPHYLOCOCCAL BACTERIOPHAGES IN HUMAN SERA

I Assay of antibodies in healthy individuals and in patients with staphylococcal infections

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Sera from healthy individuals and from patients with staphylococcal infections were examined for anti alpha lysin and for antibodies against staphylococcal phage antigens The phage antigens consisted of purified phages of the serological groups A, B and F, which were adsorbed to tanned sheep erythrocytes In healthy individuals elevated antibody titres against each of the phage antigens were found in 9-11 per cent and elevated ASta titres in 1 per cent Elevated titres against phage antigen group A were found in 71 per cent of the patients The corresponding figures for antigen group B and F were 25 and 47 per cent, respectively Elevated ASta titres were found in 44 per cent of the patients A four fold rise of the antiphage antibody titre could occur within 6-14 days in patients with acute staphylococcal infections

The need of supplementary serological tests besides the antistaphylolysin reaction (ASta-reaction) in the diagnosis of staphylococcal infections has long been felt In recent years this need has increased as staphylococci are responsible for a notable number of hospital infections The diagnostic value of the ASta-test has often been questioned as the reaction may be negative even in patients with severe infections Thus, in several investigations only 40-50 per cent of patients with verified staphylococcal infections have shown elevated ASta values (3, 5, 8, 9, 18)

Attempts have been made to demonstrate antibodies against other staphylococcal products e g leucocidin, staphylokinase and coagulase (8, 9 11 17) The antileucocidin titre seems to rise more regularly in staphylococcal infection than does the ASta titre and the use of both tests in the investigation of suspect staphylococcal infections has been recommended (9, 18) It appears, however, that the antileucocidin test has not been widely used

Bacteriophages may develop in cultures of lysogenic bacteria as a result of so-called spontaneous induction The frequency of lysogenic *Staphylococcus aureus* strains has been given as 40-95 per cent (1, 14, 15) If the infection with lysogenic staphylococcal strains in a host is accompanied by a production of phages, it is probable that an individual with a fairly deep infection will develop antibodies against the phages produced by the infecting strain Neutralizing tests have so far shown that phages attacking *Staph aureus* strains of human origin and capable of establishing lysogenic systems belong to the serological groups A, B and F (13)

It has recently been demonstrated

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TABLE 1 *Agglutination with Rabbit Antisera of Tanned Sheep Erythrocytes Coated with Staphylococcal Phages Group A, B and F, Respectively*

	TC A	TC B	TC F	Uncoated cells
anti A*	25600–51200	< 50	< 50	< 50
anti B	200	51200–102400	< 50	< 50
anti F	< 50	< 50	25600–51200	< 50

* Titres expressed as reciprocals of serum dilution

Staph aureus-phages can be grouped serologically also by indirect haemagglutination (IHA) Grouping of staphylococcal typing phages by IHA coincided completely with the serological division of these phages by neutralizing tests Of 33 phages isolated from *Staph aureus* strains of human source and grouped by IHA, 21 belonged to group A and 12 to group B (7)

This paper concerns examination in IHA of sera from healthy persons and from patients with staphylococcal infections for antibodies against phage antigen The antigen used consisted of purified phages, representing the three serological groups A, B and F

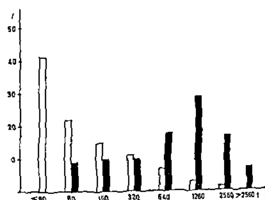


Fig 2 Distribution of the titres against phage antigen group A in serum of 100 blood donors and 68 patients with staphylococcal infections

☐ blood donors

■ patients

Titres expressed as reciprocals of serum dilution

MATERIAL AND METHODS

The material consisted of serum samples from 100 healthy persons (registered blood donors) and from 68 patients with staphylococcal infections Forty of these patients had infections of the bones or joints in 34 of them the diagnosis was osteitis or osteomyelitis Twenty eight of the patients had soft tissue infections deep abscesses and deep postoperative wound infections (21) empyema (3) and sepsis or lymphangitis (4) *Staph aureus* was isolated on various occasions from the infectious process in all patients Most of the patients received treatment with antibiotics before the blood samples were obtained The serum was stored at −20° C

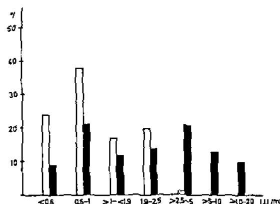


Fig 1 Distribution of anti-α staphylolysin titres in serum of 100 blood donors and 68 patients with staphylococcal infections (I U /ml)

☐ blood donors

■ patients

Anti α staphylolysin (ASta)

Staphylococcal α lysin and anti α lysin standard serum were obtained from The State Bacteriological Laboratory, Stockholm

Fresh rabbit blood cells were prepared The cells were washed and suspended to a concentration of 2 per cent in 0 15 M saline The density of the suspension was checked photometrically

To twofold dilutions of inactivated serum in 0 15 M saline with 1 per cent human albumin was added the dose of toxin which had been found to give 50 per cent haemolysis in the presence of 0 05 unit of standard anti lysin The volumes of

serum and toxin dilutions in each tube were 0.5 and 1.0 ml, respectively.

The tubes were incubated at + 37° C for 30 minutes after which 0.5 ml of the blood cell suspension was added to each tube. The tubes were then incubated again at + 37° C for 60 minutes, and afterwards at + 4° C for three hours before the results were read. The degree of haemolysis was determined and the anti-α lysin titre was calculated. Each test included two sera with known anti α lysin titres as controls.

Indirect Agglutination of Phage Coated Sheep Erythrocytes (IHA)

The antigens used consisted of phages A1, 71 and 42D, representing the serological groups A, B and F, respectively. The phages were propagated and sterile filtered as earlier described (7).

Purification of Phage Antigen for Adsorption to Tanned Erythrocytes

A sterile filtered phage suspension in Tryptone Soya Broth was concentrated 10–20 times by ultra filtration (Diaflo membrane XM 100), and then diluted to the original volume by addition of broth. This was repeated twice. After a fourth ultrafiltration broth was added to give a calculated concentration of phages of $1 \times 10^{11} - 1 \times 10^{12}$ plaque forming units (p.f.u.)/ml. The purified phage was stored at + 4° C.

Tanned phage coated erythrocytes were prepared as described earlier (7). The indirect haemagglutination test (IHA) and the indirect haemagglutination inhibition test (IHAI) were performed as previously described (7). The sera were not inactivated. Two sera with known IHA titres were always included in the tests as controls.

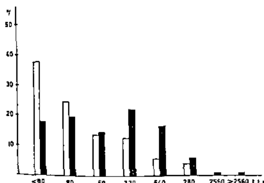


Fig 3. Distribution of the titres against phage antigen group B in serum of 100 blood donors and 68 patients with staphylococcal infections.
☐ blood donors
■ patients
Titres expressed as reciprocals of serum dilution.

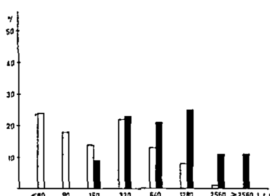


Fig 4. Distribution of the titres against antigen group F in serum of 100 blood donors and 68 patients with staphylococcal infections.
☐ blood donors
■ patients
Titres expressed as reciprocals of serum dilution.

Table 1 gives the IHA titres with the test sera against cells coated with homologous and heterologous antigen, respectively. The test sera were obtained by immunizing rabbits with phage antigen purified by gel filtration (7). When attempts were made to increase the titres by increasing the amount of antigen on the cells they agglutinated spontaneously in IHA and could not be stabilized.

The same batch of erythrocytes coated with the respective phages was used throughout the investigation. The cells were distributed in 2 ml portions and stored at + 4° C.

In those cases where heterophilic antibodies were found (positive reaction with the uncoated control cells), the sera were absorbed in the conventional way.

The statistical analysis was performed by *B. Ringner*, B. Sc., Department of Mathematical Statistics the Lund Institute of Technology, Lund.

Reproducibility of the Tests

The ASta and IHA titres in 4 sera with low and high titres, respectively, were determined on 19 occasions (different days). Statistical analysis showed a difference in ASta titre of at least 1.6 I.U. and at least a four fold change in IHA titre to be significant at the 5 per cent level.

RESULTS

Figs. 1, 2, 3 and 4 give the distribution of ASta titres and the IHA titres with cells coated with the phage antigens. The blood samples from the patients (one sample from each of the patients) were obtained

TABLE 2 *ASta titres and Titres of Anti phage Antibodies in Sera from 100 Registered Blood Donors and 68 Patients with Staphylococcal Infections*

Blood donors						Patients*					
Indirect haemagglutination§			ASta (I U /ml)			Indirect haemagglutination§			ASta (I U /ml)		
			<1 9	1 9 2 5	≧3 1				<1 9	1 9–2 5	≧3 1
Phage antigen group A	< 320	78	65	13	0	Phage antigen group A	< 320	19	14	1	4
	320	11	8	3	0		320	10	6	0	4
	> 320	11	6	4	1		> 320	71	22	13	36
Phage antigen group B	< 320	77	61	15	1	Phage antigen group B	< 320	53	26	6	21
	320	13	10	3	0		320	22	6	6	10
	> 320	10	8	2	0		> 320	25	10	2	13
Phage antigen group F	< 640	78	65	13	0	Phage antigen group F	< 640	32	21	5	6
	640	13	7	5	1		640	21	7	3	11
	> 640	9	7	2	0		> 640	47	14	6	27

* In per cent
§ Titres expressed as reciprocals of serum dilution

days after the onset of symptoms, in cases of chronic osteomyelitis, 10 to 20 days after the clinical onset of an exacerbation Assuming ASta-titres of 1 9–2 5 I U /ml as the upper limit of the normal range, 21 per cent of the sera from blood donors had a titre at or above that level When the corresponding values for the IHA reactions with TC-A and TC B were taken as 1/320 and with TC F as 1/640 22–24 per cent of the sera from the blood donors had a titre at or above those levels When titres above the upper limit were regarded as elevated, 1 per cent of the sera from blood donors had elevated ASta titres and 9–11 per cent had elevated titres against each of the three phage antigens 48 (71 per cent) of the patients' sera had elevated titres against TC A The corresponding figures for TC-B and TC-F were 17 (25 per cent) and 32 (47 per cent), respectively, and for ASta 30 (44 per cent), (Table 2)

TABLE 3 *Distribution of Patients' Sera and Sera from Blood Donors with Elevated Titres Against one or Several of the Antigens α lysin and Phage Antigens Group A, B and F**

Antigens	Blood donors§	Patients§
α lysin	1	44
Phage antigen A	11	71
Phage antigen B	10	25
Phage antigen F	9	47
α lysin + A	11	79
α lysin + B	11	56
α lysin + F	10	66
α lysin + A + B	18	87
α lysin + A + F	16	87
α-lysin + A + B + F	23	89
Phage antigens A + B	18	82
Phage antigens A + F	16	82
Phage antigen A + B + F	23	88

* Titres above the chosen upper limit of the normal range were regarded as elevated
§ In per cent

On comparison of blood donors and patients the statistical analysis showed that the number of patients with elevated titres against TC-A and TC F was significantly higher than that of patients with elevated titres against TC B The number of patients with elevated ASta titres was also significantly higher than that of those with elevated titres against TC-B No significant difference could be found between TC-A and ASta

The patients with elevated titres against TC A (48 of 68) included 9 of the 17 and 24 of the 32 patients with elevated titres against TC B and TC F, respectively Among the same 48 patients were also found 24 of the 30 patients with elevated ASta values

TABLE 4 *ASta titre and Titre of Anti phage Antibodies in Sixteen Patients with Acute Staphylococcal Infections*

Patients	I*	II§	Phage antigens†			ASta†
			A	B	F	
1 Epidural abscess	ca 8	6	160–640	320–1280	320–640	0 9–0 9
2 Empyema	8–10	7	320–320	<80 <80	<80–320	0 9 1 6
3 Septicemia	2	6	80 80	80 80	320–1280	1 25–12 0
4 Spondylitis	6	7	80–640	<80 <80	160–320	0 6–0 9
5 Post operative wound infection	10–14	11	80–640	160–160	640–1280	3 1–4 4
6 Lymphangitis	4–5	6	320–320	320–1280	80–640	1 0–1 0
7 Deep abscess	12–14	9	1280–5120	640–320	1280–10240	1 0–7 5
8 Lymphangitis	9	14	80–640	<80–<80	160–320	0 6–0 6
9 Purulent arthritis	5	20	80 80	<80–<80	160–<80	0 6–0 9
10 Post operative wound infection	9	14	320–80	<80 <80	2560–160	0 75–1 0
11 Nasal furunculosis	8–10	7	2560–640	320–320	320 320	1 9–1 6
12 Empyema	8–10	20	1280–640	2560–1280	2560–2560	17 5–15 0
13 Submandibular abscess	ca 14	10	1280–1280	<80–<80	<80–<80	2 2–2 2
14 Purulent arthritis	5	20	80–80	<80–<80	80 80	15 0–20 0
15 Post operative wound infection	3	22	<80–<80	<80–<80	80–160	1 25–1 6
16 Post operative wound infection	15	14	<80–<80	<80–<80	<80–<80	0 9–0 9

* I Number of days between onset of symptoms and first serum sampling
§ II Number of days between first and second sampling
† ASta titre and titre in IHA in first and second serum sample ASta I U /ml IHA titres expressed as reciprocals of serum dilution

Thus, only 6 patients (8 per cent) had elevated ASta titres without an elevated titre against TC A The corresponding figures in comparison of ASta-titres with the titres against TC B and TC F were 21 (31 per cent) and 12 (17 per cent), respectively, (Table 2)

Patients with strongly elevated ASta titres (above 10 I U /ml) had practically always strongly elevated IHA titres (> 1/2500) against at least one of the phage antigens

Table 3 compares the number of sera from blood donors and patients with elevated titres against one or several of the four antigens In IHA with TC A 71 per cent of the patients showed elevated titres in comparison with 11 per cent of the normal individuals With all the four antigens the number of normal individuals and of patients with elevated titres against one or several of the antigens increased to 23 and 89 per cent, respectively

In 7 of the patients' sera (11 per cent) no elevated titres were found against any of the four antigens Three of these sera had titres at the upper limit of the normal range against one or more than one of the phage antigens

No difference in distribution of the titres against the four antigens was found between sera from patients with soft tissue infections and patients with infection of bone or joints

In order to detect changes in IHA- and ASta titres 16 of the patients with acute infections were examined at least 2 times during the first month after onset of symptoms (Table 4) In 8 of these patients (Nos 1–8) at least a four fold rise in IHA titre occurred against one or two of the phage antigens in 6–14 days In three patients (Nos 9–11) at least four fold fall in titre was fo

TABLE 5 *Inhibition of Agglutination of Erythrocytes Coated with Staphylococcal Phages Group A, B and F in Tests with 5–10 Agglutination Units of Human Sera**

Phage preparations used as inhibitors	Titre of phage preparations§	Titre of phages giving inhibition§	
A1	80	1–8	
A1	2 0	1–2	Sera with titre against TC A vs TC A
71	70	> 70	Number of sera = 23
42D	50	> 50	
A1	80	> 80	
71	70	2–8	Sera with titre against TC B vs TC B
71	4 0	2–4	Number of sera = 11
42D	50	> 50	
A1	80	> 80	
71	70	> 70	Sera with titre against TC F vs TC F
42D	50	0 5–4	Number of sera = 24
42D	1 0	0 5–1	

* Ten sera from normal individuals and 21 sera from patients were investigated Five of the sera had titres against both TC A, TC B and TC F, 17 had titres against two and 9 against one of the antigens
§ Plaque forming units × 10^9/ml

7–20 days Elevated IHA-titres without significant rise or fall were found in 2 patients (Nos 12–13) In the remaining 3 patients (Nos 14–16) no titre or low titres without significant changes were observed

In three patients (Nos 2, 3 and 7) an at least 1 6 fold rise in ASta titre was found while three patients (Nos 5, 12 and 14) had elevated ASta-titres without significant changes

Twenty of the patients with chronic osteomyelitis were investigated further during exacerbation and regression of the infection The results will be reported in the next paper (6)

Specificity of the IHA Reactions

The phages used as antigens were sterile filtered and then concentrated and purified by ultrafiltration Thus, it was not excluded that the IHA also could involve reactions between antibodies and traces of constituents or products from the propagating strains which had passed the sterile filter and were retained by the ultrafiltration membrane Protein A could not be detected in the purified phage preparations when examined by the method of *Forsgren* (2)

If the reactions in IHA were specific for each of the three antigens, the agglutination should not be inhibited by heterologous phage Further, the capacity of a phage suspension to inhibit in the homologous system should be correlated with the number of p f u /ml A preparation with a low phage titre and a preparation with a high yield of the same phage, propagated on the same bacterial strain should therefore be expected to inhibit at the same number of p f u /ml

It is wellknown that propagation of phages in soft agar sometimes gives an unexpectedly low yield even if the conditions are kept constant By the use of preparations with low yields, phage suspensions were obtained which probably contained traces of constituents and products from the propagating strains in at least the same amount as preparations with a high yield of phages

One preparation with a high and one preparation with a low titre of each of the three phages were used as inhibitors in a series of inhibition tests The preparations were sterile filtered but not concentrated or purified

Inhibition tests were carried out with 33 sera 22 of these had antibodies against two or three of the antigens

The results are given in Table 5 No inhibition appeared in the heterologous systems In the homologous systems agglutination was inhibited by phages in titre of 0 5–8 × 19⁹ p f u /ml In some cases the number of p f u / ml in the preparations with low phage titre was too small to produce any inhibition

DISCUSSION

Judging from the results of the inhibition tests it was unlikely that the IH tests with phage-coated erythrocytes represented antigen antibody reactions other than those specific for each of the three phage antigens It is not known whether there are related antigens among staphylococcal phages and constituents products or phages of other bacterial genera This is unlikely considering the striking antigenic specificity of the different groups of the staphylococcal phages

As *Staph aureus* normally can be found in humans for instance in the upper respiratory tract antibodies against staphylococcal constituents and products appear in serum of healthy individuals (9 10) The presence of *Staph aureus* in the troat seems to be able to stimulate the production of anti α lysin while carriers of the bacterium in the nose usually not have elevated ASta titres (3 12)

The ASta values of sera from healthy individuals found in the present investigation corresponded well with those reported by a large number of investigators For a review see *Harter et al* 1965 (4)

Judging from the results obtained on comparison of healthy individuals and the 68 patients in whom one serum sample was examined 10–20 days after the onset of symptoms the IHA test with antigen A was more sensitive than the ASta test With antigen F largely the same result was obtained as with the ASta test which was significantly more sensitive than IHA with antigen B The frequency of patients with elevated ASta titres 44 per cent agreed with other authors results (3 5 8 9 18) On the other hand the ASta test was more specific than the reactions in IHA with elevated titre in only one per cent of the healthy individuals sera in comparison with 9–11 per cent for each of the phage antigens

Most of the patients sera with elevated ASta values and elevated titres against antigen F also had elevated titres against antigen A Among the sera with elevated anti A titres also more than half of those with elevated titres against antigen B were found Thus the combined use of the antigens gave a relatively slight increase in the number of patients sera with elevated titres At the same time the number of sera from healthy individuals with elevated antibody titres against the phage antigens increased in about the same number (Table 3)

However for the serological diagnosis in the single patient it seems to be of greater interest to examine several serum samples than to compare the titre of one serum sample with the distribution of the titres in a population of healthy individuals

In 8 out of the 16 patients with acute staphylococcal infections who were examined for titre changes during the first month after the onset of symptoms a significant rise in IHA titre occurred against one or two of the phage antigens in 6–14 days (Table 4)

It is probable that the antibiotic treatment that started in all patients a the time when the first serum sample was collected suppressed the antigenic stimulation at least in some cases The fact that a significant titre fall occurred in 3 patients who all responded well to therapy indicates that a rapid fall in IHA titre can be expected in some patients when the infection regresses

In 13 out of the 16 patients the information obtained by the IHA test could be regarded as useful (significant titre rise in 8 patients significant titre fall in 3 and elevated titres without changes in 2 patients) The ASta reaction gave the same information in 6 patients (significant titre rise in 3 patients and elevated titres without changes in 3 patients) In no case was a significant fall in ASta titre observed

Thus the results suggest that the IHA with purified phages as antigens would be ι

as a serological test in staphylococcal infections if repeated tests are done For this purpose the combined use of antigens A and F seems to be suitable, while antigen B appears to be of little interest

The rise and fall of the antibody titre in the same patient against more than one of the phage antigens was in agreement with the fact that a *Staph aureus* strain can be lysogenic with phages of different serological groups (7, 16)

Staphylococcal strains of human source are often lysogenic with group B phages (7) The antibody titres in the sera from healthy individuals showed largely the same distribution against all the three phage antigens A difference in quality of the phage coated erythrocytes or a decreased ability in humans to produce antibodies against antigen B therefore do not explain the low frequency of patients' sera with elevated titre against this antigen

A further evaluation of the test is reported in the next paper (6)

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ANTIBODIES AGAINST STAPHYLOCOCCAL BACTERIOPHAGES IN HUMAN SERA

II Assay of antibodies in exacerbation and regression of chronic staphylococcal osteomyelitis

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Sera from 20 patients with chronic staphylococcal osteomyelitis were examined in respect of ASta titres and antibody titres against staphylococcal phage antigen during exacerbation and regression of the infection The antiphage antibodies were determined by indirect haem agglutination (IHA) In 13 of the patients a correlation was found between the IHA titre and the clinical course, i e a titre rise during exacerbation and a titre fall during regression while the ASta reaction showed the same correlation in 5 cases In 4 patients no titres could be demonstrated in IHA, while 3 constantly had high titres, irrespective of the clinical course

By indirect haemagglutination test (IHA) with phage coated sheep erythrocytes, it has been shown that antibodies against staphylococcal phage antigens group A, B and F (3) occur in both healthy individuals and in patients with staphylococcal infections (4) In a series of 16 patients with acute staphylococcal infections it was found that a four-fold increase of the titre of antiphage antibodies could occur within 6–14 days It was suggested that the IHA test would be of benefit as a serological test in staphylococcal infections and that repeated tests should be done in order to detect titre changes (4)

Twenty patients with chronic osteomyelitis were examined further during exacerbation and regression of the infection The results are given in the present paper

The purpose was to compare ASta-titres and titres in IHA with the clinical course and to evaluate the use of these tests in the estimation of the activity of the infection

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MATERIAL AND METHODS

Patients

The material consisted of 20 patients who had had osteomyelitis of the long shaft bones or hip bones for at least 6 months The diagnosis was established roentgenographically and/or patho-anatomically

Staphylococcus aureus was isolated from the focus of the infection either at the time of the actual exacerbation or on some earlier occasion Apart from osteomyelitis, the patients had no active, serious disease and were not being treated with steroids or other immunosuppressive therapy

In the clinical evaluation of the activity of osteomyelitis notes were made at every examination of symptoms (pain, malaise), fever, local signs of inflammation (swelling, reddening, increased skin temperature, tenderness to palpation) and secretion from fistulae Roentgenological examination was not routinely performed in all the cases

Signs and symptoms and laboratory data (E S R

TABLE 1 *Rise and Fall of ASta- and IHA titres during Exacerbation and Regression, respectively, in Thirteen Patients with Chronic Staphylococcal Osteomyelitis*

Patients	Days*	Titre rise on exacerbation†				Titre fall on regression			
		anti A	anti B	anti F	ASta	anti A	anti B	anti F	ASta
1	14	4	–	2	0 6–3 7	16	–	8	4 4–2 2
	19	16	–	2	0 6–4 4				
2	28	–	4	2	Negative	–	32	64	Negative
3	17	–	4	4	Negative	–	4	2	Negative
4	c 60	4	8	4	Negative	No fall	4	4	Negative
5	21	4	8	8	Negative	4	8	16	Negative
6	18	32	–	32	Negative	32	–	32	Negative
7	24	–	4	4	Negative	–	8	4	Negative
8	9	4	–	4	5 0–5 0	8	–	4	5 0–3 7
9	c 30	4	–	4	Negative	32	–	16	Negative
10	10	No rise	–	4	Negative	No fall	–	4	Negative
11	c 30	2	–	4	2 2–3 1	2	–	4	3 1–1 6
12	c 90	Not investigated				8	4	4	5 0–2 2
13	c 35	Not investigated				–	–	4	15 0–3 1

* Number of days between beginning of exacerbation and time of the first serum sample after beginning of exacerbation From patient No 1 two serum samples were obtained at five days interval
† Compared with titre in the serum test before the exacerbation The figures for the antiphage antibodies denote 2 , 4 etc -fold titre rise and fall, respectively – <1/40 The figures for ASta denote the titre in I U /ml serum Negative ASta titre <2 5 I U /ml without significant change of titre during exacerbation or regression

and C reactive protein) were used for the estimation of the activity of the infection

Laboratory Tests

The IHA test with phage coated sheep erythrocytes and the ASta test were performed as in the previous investigation The same batches of phage coated cells were used Evaluation of the reproducibility of the test showed that a difference in ASta titre of at least 1 6 I U and at least a four fold change in the IHA titre were statistically significant (4)

C reactive protein was measured by electroimmuno assay (5)

The serum samples were stored at –20° C Repeated thawing and freezing was avoided For the IHA tests non inactivated sera were used

RESULTS

Out of the twenty patients, 13 had a titre rise in IHA during active infection and/or a titre fall on regression of the infection (*Table* 1)

Eleven of them showed at least a four-fold titre rise to one or several of the phage antigens during the exacerbation In 2 cases, Nos 12 and 13, no serum samples were obtained before the actual exacerbation The titres of the antiphage antibodies in the first serum sample obtained after the beginning of the exacerbation were 1/20,000 and 1/320, respectively

In all the 13 patients at least a four-fold

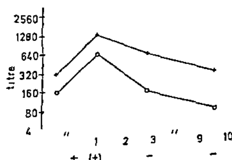


Fig 1 IHA titre during exacerbation and regression (pat No 7) Titres are reciprocals of serum dilution O titre B + titre to antigen F ASta t Titre to antigen A <1/40 + De of the infection as estimated by Material and Methods.

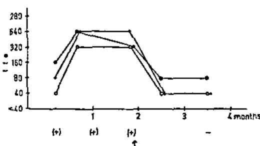


Fig 2 IHA titre during exacerbation without signs of regression before sequestrectomy (pat No 5) Titres are expressed as reciprocals of serum dilution ● titre to antigen A ○ titre to antigen B + titre to antigen F ASta titre <2 5 I U Arrow indicates time for sequestrectomy + Degree of activity of the infection as estimated by criteria given under Material and Methods

fall in titre of antiphage antibodies was demonstrated during treatment The fall was usually slow and followed the clinical regression (Fig 1) The most rapid fall was noted in 2 patients (Nos 2 and 5) in whom sequestrectomy was performed In these an eight- to sixteen fold titre fall in IHA was noted within 10 and 18 days after the operation respectively (Fig 2)

Antibodies against the different phage antigens generally showed a concordant rise and fall, respectively Exceptions were noted in patient No 4, in whom no fall in antibody titre to phage antigen group A occurred during the regression and in patient No 10, in whom the titre of antibodies to this phage antigen did not change during exacerbation or during regression

Four of the 13 patients with significant titre changes in IHA, also had a significant rise and/or fall in ASta titre (Nos 1 11, 12 and 13) (Table 1) One patient (No 8) had elevated ASta titre without significant changes In the remaining 8 patients ASta values <2 5 I U /ml were found without titre changes during exacerbation or regression of the infection

Three patients had high titres (>1/2560) to one or more phage antigens both before and during the exacerbation and no fall could be demonstrated during regression In one patient there was a significant rise in ASta titre (6 2 – 10 0 I U /ml) within 8 days after the onset of the exacerbation A titre of about 10 I U /ml persisted without any tendency to fall despite clinical regression The other 2 patients showed a shift in ASta titre during the entire exacerbation and subsequent regression which was not significant (1 6 – 2 2 units)

In 4 patients no antiphage antibody titres could be demonstrated (titre<1/40) In one of the patients a significant rise in ASta titre occurred (2 5 – 7 5 units), but this rise did not occur until 3 weeks after the patient began to show signs of regression and when no other laboratory or clinical signs suggested activity In the remaining three patients the ASta values were constant, 0 75, 0 9 and 2 5 I U, respectively, both during activity and regression

DISCUSSION

The findings in the present investigation confirmed the wellknown experience that staphylococcal osteomyelitis is accompanied by an elevated ASta-titre in less than half of the cases (2, 6, 7, 9) This may be because some strains do not produce α lysin or because the production of lysin is inhibited *in vivo* (1, 6, 8) The inability of individuals to produce antitoxin appears less likely in most cases because immunization with toxoid nearly always results in a marked antibody response (6)

In 13 of the present 20 patients a correlation was found between the titre in IHA and the clinical course, *i e* a significant titre rise during exacerbation and a significant titre fall during regression, while the ASta titre showed the same correlation in 5 cases In 2 of the patients did a significant rise occur in ASta titre without any demonstrable rise in IHA-titre In one of those patients the IHA titre was constantly high (>1/2560) irrespective of the clinical course In the other patient (IHA titre<1/40) the rise of ASta titre did not correlate with the activity of the infection

Thus, the results in the present investiga-

tion, like those of the previous report, indicated that in patients with active staphylococcal infections a significant titre rise in the IHA test is more common than a rise of the ASta-titre and that the IHA-test often is useful in the estimation of the results of treatment in many cases since a fall in titre occurred when the infection was regressing

An eight- to sixteen-fold fall in titre was observed in about a fortnight in one patient with an acute infection who responded well to therapy (4) and in two patients in the present investigation in whom sequestrectomy was performed This indicates that the antiphage antibodies reacting in the test may be of IgM-class not only in acute infections but also in such long standing conditions as chronic osteomyelitis

Judging from the results of the previous and present investigations little is to be gained from the use of antigen B in addition to the combined use of antigens A and F

In three cases the IHA-titres against one or several of the phage antigens were constantly high, irrespective of regression It is well-known that some individuals may have long-standing antibody titres for years after the actual infection

The strains from the four patients which did not show any titre against the phage antigens were unfortunately not saved It could therefore not be checked whether those strains were lysogenic or not

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THE SIGNIFICANCE OF AUSTRALIA-ANTIBODY IN BLOOD DONORS

Study of Liver Histology in Donors and Post-Transfusion Hepatitis in Recipients

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Systematic screening by immuno-osmophoresis of 7,500 consecutively registered Danish voluntary blood donors revealed 22 with Australia antibody None of these persons had a previous history of hepatitis and only three had ever received a blood transfusion In all the donors, except one, the antibody persisted during the entire period of investigation (at present 34-38 months) Liver biopsies were performed in all 22 donors No changes such as those seen in viral hepatitis or sequelae after viral hepatitis, i e fibrosis or cirrhosis were found in any of the biopsies It is suggested that stimulation of Au antibody production in the present series of donors might have been caused by cellular antigens showing cross-immunity with Au antigen and released by mechanisms other than viral hepatitis A retrospective study was performed comprising 12 recipients of blood transfusions from the donors with Au antibody None of these recipients developed post transfusion hepatitis, and in no case could Au antigen or Au antibody be demonstrated

Large amounts of Australia (Au) antibody, viz amounts detectable by serum immuno-electro osmophoresis or double immuno diffusion, are generally assumed to occur only after repeated stimulation by the Australia-antigen (*Holland & Walsh* 1969, *Blumberg et al* 1965) Accordingly, an essential part of persons carrying large amounts of Au antibody have presumably been stimulated either passively by multitransfusions with blood products containing Au antigen, or actively during repeated attacks of Au antigen positive acute hepatitis Since transfusion of Au antigenaemic blood may sometimes be correlated with transmission of infective SH virus, this might lead to both passive and active immunization against Au antigen Consequently, an increased frequency of sequelae after SH-virus infection might be expected in persons carrying large amounts of Au antibody In the first part of the present study, Danish blood donors carrying Au-antibody were examined for the presence of liver disease, particularly sequelae after previous viral hepatitis The observation that Au-antibody might indicate the simultaneous presence of Au antigen in antibody-antigen complexes formed the basis for the second part of the study where recipients of blood from donors carrying Au antibody were studied for the occurrence of post transfusion hepatitis (*Almeida & Waterson* 1969, *Shulman & Barker* 1969)

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MATERIALS AND METHODS

The first part of the series comprised 22 donors carrying Au antibody, found by systematic screen

ing of 7,500 consecutively received volunteer blood donors from the Copenhagen area Screening for Au antibody was performed by immunoelectro osmophoresis as described previously (*Banke et al* 1971) Identity reactions were performed with Ouchterlony's double immunodiffusion technique and several reference sera (*Reinicke & Nordenfelt* 1970) In four cases, the sera had to be con centrated four to five fold before an identity re action could be obtained In two cases too small amounts of Au antibody prohibited the use of the Ouchterlony technique even after concentration The Au antibody containing sera were also tested for Au antigen by immunoelectro-osmophoresis (*Banke et al* 1971)

All antibody carrying donors underwent several clinical and laboratory examinations during the period of investigation which at present has covered 34–38 months The following biochemical tests were made systematically Haemoglobin, ESR, WBC, serum bilirubin, alkaline phosphatase, prothrombin time, urine glucose and protein Serum glutamic oxalacetic transaminase (SGOT) and immunoglobulin (IgG, IgA and IgM) estimations were performed repeatedly Liver biopsy by the Menghini technique was performed in the second *to third week after detection of the Au-antibody* Assessment of the biopsies was made as described previously, without knowledge of the clinical data, employing 50 60 sections and 7 different stainings for each biopsy (*Reinicke et al* 1972 a)

The histological changes were classified in three groups according to the extent of inflammation of the portal tracts

Group I No inflammation
Group II Slight inflammation of a few tracts
Group III Slight inflammation of most tracts

The second part of the series comprised 12 pati ents who had received blood from the donors in cluded in the first part of the series This part of the study was retrospective and designed so that each time Au antibody was discovered in a donor, the recipients of the donor s blood during a three year period before detection of the antibody were traced and studied Consequently it is not known with certainty whether the donors had Au antibody in their blood at the time of the blood donation At the time of investigation six blood recipients had died, and only their records from hospitals and attending physicians were available Six recipients were alive and were examined both clinically and serologically All these persons had been transfused within two years of detection of donor Au antibody

RESULTS

Blood Donors

Of the 22 donors carrying Au antibody, 13 were males and 9 females Thirteen of the donors were over 30 years of age The Au antibody persisted in all donors, except one, during the entire period of investigation (at present 34–38 months) Au antigen was not detected in any of the donors

None had any clinical signs of disease and the results of routine laboratory examinations were normal in all instances

The main histological findings are shown in Table 1 In no case did infiltration of portal tracts or lobules by lymphocytes and plasma cells exceed a slight degree Neither fibrosis nor cirrhosis was found in any case All donors had normal SGOT values re peatedly, except the donor with steatosis in whom a two to three-fold elevation was found

IgM and IgA values were normal in all instances A slight increase of IgG values ($\leq$ 18 1 g/litre) was found in seven of the sixteen donors in group I Three of these had normal parenchyma Two showed a few fat droplets and the remaining two were the donors with moderate steatosis and slight parenchymal inflammation One donor in group II and one in group III also showed slight IgG increases

None of the donors had a history of previ ous hepatitis or drug addiction Only three had ever received blood transfusion with one five and one units, respectively None had been treated with cytostatic drugs and, ex cept for two who were taking The Pill none had been given steroids None of the donors had ever been in the tropics One donor was an alcoholic and showed moderate steatosis by liver histology Recovery from diseases of childhood and minor respiratory illnesses seemed to have been normal in all donors

Sixteen of the donors had been hospital ized, three for hysterectomy, one for a goitre operation, one for an extra uterine pregnan cy, and the remainder for observation minor surgery or fractures

Blood Recipients

The 12 recipients had received blood from six of the 22 donors with Au antibody (*five group I donors, and one group II donor*)

TABLE 1 *Main Histological Findings in Liver Biopsies from 22 Clinically Healthy Au antibody Carrying Blood Donors*

Group	No of donors	Portal tracts	Lobules
I	16	No inflammation	7 normal histology (2 slight haemosiderosis) 7 few fat droplets 1 moderate steatosis 1 slight inflammation
II	3	Slight inflammation of a few tracts	1 normal histology 1 few fat droplets *1 thorium dioxide*
III	3	Slight inflammation of most tracts	1 normal histology 2 slight inflammation (1 slight haemosiderosis)

Blood from the remaining 16 donors had never been used for transfusion

The six patients who were alive at the time of investigation had no history of acute hepatitis Normal serum GO transaminase levels were found and neither Au antigen nor anti body could be demonstrated

The six patients who died (one from cancer of the colon two from chronic pyelo nephritis and uraemia, one from alcoholic cirrhosis with hepatic failure, and two from arteriosclerotic heart disease with cardiac failure) had no history of hepatitis following the transfusion No macroscopical evidence of hepatitis could be found on autopsy in these six cases

DISCUSSION

Seven of the 22 liver biopsies of donors with Au antibody were completely normal (group I) Seven biopsies showed a few fat droplets and may also be considered as normal (group I) Small numbers of lymphocytes and plasma cells were found in some of the portal tracts of three donors (group II) These changes are non specific and may be seen in conditions such as gall stones and gastric ulcer However similar changes may be late sequelae after previous viral hepatitis (*Bianchi et al* 1971) In one case the changes were seen together with deposits of thorium dioxide This patient had had a thorium dioxide angiography performed many years previously

Although very slight, the changes found in the three group III donors are not entirely incompatible with a histological diagnosis of chronic persistent hepatitis (*De Groote et al* 1969) However, the clinical and biochemical findings do not support that diagnosis

None of the biopsies from the 22 Au anti body carrying donors showed signs of acute hepatitis, chronic aggressive hepatitis or cirrhosis

None of the donors had been treated with cytostatic drugs and with the exception of two women who took The Pill, none had received steroids None had a history of hepatitis or drug abuse Only three donors had ever received a blood transfusion, one of these repeatedly (5 units) Thus, there is no evidence of repeated Au antigen exposure in the majority of the donors

No significant signs of chronic liver disease were found in any of the donors, although this possibility cannot be entirely excluded in the case of the three group III donors These results agree with the findings by *Klinge et al* (1971) and *Iwarsson et al* (1972), who also investigated the liver histology of donors with Au antibody

The apparent lack of repeated exposure to hepatitis and the lack of predominance of

chronic hepatitis among the donors would perhaps suggest that the repeated antigen stimulation of donors carrying Au antibody might have been caused by cellular antigens showing cross immunity with Au antigen The antigens might have been released by conditions entirely different from viral hepatitis, such as, for instance alcoholism This hypothesis is supported by the finding that signs of previous Au antigen stimulation have been described in patients with alcoholic cirrhosis (*Pettigrew et al* 1972) There are also other possibilities for repeated antigen stimulation, since Au antigenaemia has been observed in several conditions where there was no apparent relaton to viral hepatitis (such as L E D, polyarteritis, and leprosy) Also non specific stimulation with inactivated foot-and mouth disease virus seems to raise the serum titre of Au antigen in individuals with Au-antigenaemia (*Aron & Maupas* 1973)

No marked changes in immunoglobulin and SGO transaminase levels were found in any of the present donors, and the few small IgG elevations distributed equally in all the histological groups are of doubtful significance Although unproved, some of the transfusion blood from these donors probably contained Au antibody, since the Au-antibody carrier state up to now has been lasting for 34–38 months No case of transfusion hepatitis could be found among the 12 blood recipients Thus, the postulated risk of infective Au antigen/Au-antibody complexes in blood containing Au antibody has not been supported by the present findings This is in agreement with our previous observations that no Au antibody carriers could be found retrospectively among the blood donors of recipients with verified post transfusion hepatitis (*Reinicke et al* 1972 b)

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CROSS-REACTIVITY BETWEEN HEAT LABILE ENTEROTOXINS OF *VIBRIO CHOLERAE* AND *ESCHERICHIA COLI* IN NEUTRALIZATION TESTS IN RABBIT ILEUM AND SKIN

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Crude enterotoxin preparations of porcine enteropathogenic *Escherichia coli* were found to be active both in the ileal loop model and in the intradermal test in rabbits, as was crude and isolated exo-enterotoxin of *Vibrio cholerae* The skin activity of the *E coli* enterotoxic preparations was destroyed by incubation at 65° C for 10 minutes Neutralization studies in the ileal loop and the intradermal tests indicated that the heat labile enterotoxins produced by *E coli* of different O groups shared antigenic determinants to a varying extent, and also showed some immunological cross reactivity with the cholera exo-enterotoxin

The enterotoxins produced by the two main serotypes of *Vibrio cholerae (V cholerae)*, Inaba and Ogawa, have been shown to be immunologically identical (*Holmgren et al* 1971) Enterotoxins from enteropathogenic strains of *Escherichia coli (E coli)* isolated from pigs and human subjects were described a few years ago (*Smith and Halls* 1967, *Kohler* 1968, *Gyles & Barnum* 1969, *Gorbach* 1970, *Sack et al* 1971, *DuPont et al* 1971) Knowledge of similarities and differences in the structure and action of the cholera exo enterotoxin and of the enterotoxins produced by various *E coli* strains is only fragmentary This includes immunological information where the few studies performed report conflicting results (*Gyles and Barnum* 1969, *Sack et al* 1971 *Gyles* 1971, *Smith* 1972) This study was initiated to clarify whether the enterotoxins of *V cholerae* and *E coli* share structures demonstrable by cross neutralization analyses

During the course of our study, it was shown by *Smith and Sack* (1973) that enterotoxins produced by *E coli* from human diarrhoea cases were immunologically similar but not identical with the enterotoxin produced by *V cholerae* The present study shows that this cross-reactivity extends to the heat labile enterotoxins produced by *E coli* strains causing diarrhoea in pigs It is also demonstrated that the *E coli* enterotoxins can cause increased capillary permeability in the skin of rabbits, which considerably scales up the bioassaying of these toxins

MATERIALS AND METHODS

Enterotoxins Isolated cholera toxin choleragen from *V cholerae* serotype Inaba was prepared as described (*Finkelstein and LoSpalluto* 1970) under contract for the National Institute of Allergy

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Infectious Diseases by *R A Finkelstein** and had the lot no 1071 The freeze dried material was used dissolved in 0 1 M Tris HCl buffer, pH 7 5, containing 0 2 per cent (w/v) gelatin for stabiliza tion (TG buffer)

Crude *V cholerae* (Inaba 569 B) enterotoxin was also provided by the National Institutes of Allergy and Infectious Diseases, Bethesda, Md as freeze dried material It was dissolved in TG buffer before use, 25 mg/ml corresponds to the original culture filtrate concentration

Crude enterotoxins from *E coli* were prepared from 5 strains isolated in Sweden from pigs in herds with acute enteric disease (*Söderlind* 1971 a and b) and 1 porcine enteropathogenic strain P5 (O 141), received from Dr *H W Smith*** The strain designations of the Swedish strains—the somatic O antigens within parentheses—were 915/66 (O 8), 163/67 (O 138), B 2577/67 (O 147), 537/67 (O 149) and 853/67 (O 149)

Roux bottles containing 200 ml of tryptose agar (Tryptose Phosphate Broth, Oxoid, 29 5 g, Bacto agar, Difco, 15 g, de ionized water 1000 ml) supplemented with 0 2 per cent (w/v) glucose, were inoculated with 5 ml of an 18 h old culture of the bacterial strain in nutrient broth After incubation at 37° C for 18 h the organisms were harvested by shaking and washing the cells from the agar with 20 ml distilled water containing 12 g of glass beads per bottle The bacterial suspension (approx 3×10^{10} viable cells per ml, approx 30 mg dry weight per ml) was subjected to ultrasonic treatment (MSE, 60 W ultrasonic dis integrator, Measuring Scientific Equipment Ltd, London) at 10 Kc/sec, 1 3 A and 10 μ amplitude in 12 ml batches for 5 min After 3 hours' centri fugation ($4000 \times g$) the clear supernatant was filtered through 0 65 μ and 0 45 μ cellulose acetate filters (Millipore) and neomycin was added to a final concentration of 100 μg/ml The dry weight of this material was approximately 30 mg per ml

The enterotoxins from P5 and 853/67 were stored in aliquots at 20° C until use within one month while the other *E coli* enterotoxin pre parations were lyophilized and kept at 4° C until use up to one year later when they were dissolved in the TG buffer for each test occasion Two toxin preparations from different cultures were used from strain B 2577/67, I and II

Antisera Antiserum to cholera toxin was ob tained from a rabbit given three subcutaneous in jections of 30 μg of the purified toxin without adjuvant three weeks apart Bleeding was per formed two weeks after the second injection

Antiserum against enterotoxin from *F coli* strain P5 was produced in a rabbit given three sub cutaneous injections of the partially purified enterotoxin three weeks apart (*O Söderlind, R Möllby and T Wadström* Unpublished) On the first two occasions, 1 ml of enterotoxin containing 1 25 mg of protein from a fraction (pH 4 15) ob tained after isoelectric focusing of crude entero toxin which had been treated with ammonium sulphate at 65 per cent saturation and the pre cipitate redissolved was injected On the third occasion, 1 ml of the same material before iso electric focusing was injected Bleeding was per formed 10 days after the last injection

Enterotoxin Assays and Neutralization

For the ileal loop tests 6–9 weeks old rabbits were used For the skin assays the rabbits were 14–20 weeks old Neutralizing capacity of antisera was tested by incubating equal volumes of ap propriate dilutions of enterotoxin with dilutions of antiserum at 37° C for 30 min followed by an assay of toxic activity of the mixture in the ileal loop model (*De & Chatterje* 1953) or in the intra dermal test (*Craig* 1965) These two bioassays were performed as previously described with the modifi cation that the terminal 70 cm of ileum were rinsed with 20 ml of saline before the loops were pre pared (*Holmgren et al* 1973 b)

All dilutions were made in TG buffer In the ileal loop determinations of toxic activity of the *E coli* preparations, a positive control consisting of 3 μg or 500 ng of isolated cholera toxin was included in each animal together with a negative control consisting of TG buffer or cholera toxin mixed either with G_{M1} ganglioside (*Holmgren et al* 1973 a, b) or with the homologous anti cholera toxin antiserum For toxicity determinations by means of the intradermal assay similar controls were included with different concentrations of cholera toxin In the neutralization assays in ileum and skin tests, the positive control consisted of the enterotoxin dose mixed with TG buffer instead of antiserum, and the negative control(s) of TG buffer and/or a mixture of cholera toxin and G_{M1} ganglioside Data from animals which did not respond properly to these controls are not included in the presentation of results approximately one third of the animals belonged to the excluded category

Heat stability Serial dilutions of the *E coli* enterotoxins in the TG buffer were kept at 65° C in a water bath for 10 min and were then tested for toxic activity in the intradermal assay Such heating has been reported to destroy the heat labile *E coli* enterotoxins but not the heat stable toxin (*Gyles* 1971)

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TABLE 1 *Reactivity of* V. cholerae *and* E. coli *enterotoxins in Ileal Loop and Skin Tests*

	Toxicity	
	Ileal loop *	Skin †
Isolated *V. cholerae* 569 B (Inaba)	+ (200 ng/ml)	+ (2 ng/ml)
Crude *V. cholerae* 569 B (Inaba)	+ (100 µg/ml)	+ (10 µg/ml)
Crude *E. coli* 915/66 (O group 8)	+	+ (1/10)
Crude *E. coli* 163/67 (O group 138)	—	+ (1/5)
Crude *E. coli* P 5 (O group 141)	+ (1/8)	+ (1/10)
Crude *E. coli* B 2577/67 (O group 147) I	+	+ (1/160)
Crude *E. coli* B 2577/67 (O group 147) II	+	+ (1/65)
Crude *E. coli* 537/67 (O group 149)	—	+ (1/2)
Crude *E. coli* 853/67 (O group 149)	+ (1/20)	+ (1/200)

* + indicates fluid accumulation > 1.0 ml per cm of gut and — indicates absence of fluid accumulation as tested with 2 ml of 1/2 dilutions of the crude enterotoxin preparations of *V. cholerae* or *E. coli* and with 1.5 µg/ml of isolated cholera toxin. Values within parentheses are limiting dilutions for a + reaction.

† + indicates a skin reaction ≥ 7 mm in diameter as tested with 0.1 ml enterotoxin solution. Values within parentheses are limiting dilutions for a + reaction.

TABLE 2 *Neutralization of* V. cholerae *and* E. coli *Enterotoxins with Antisera in Ileal Loop and Skin Tests*

	Neutralizing titres			
	Anti *V. cholerae* toxin		Anti *E. coli* toxin	
	Ileal loop*	Skin†	Ileal loop*	Skin†
Isolated *V. cholerae* 569 B (Inaba)	500§	12 500	5	30
Crude *V. cholerae* 569 B (Inaba)	450	12 000	NT$	30
Crude *E. coli* 915/66 (O group 8)	< 20	NT	< 20	NT
Crude *E. coli* 163/67 (O group 138)	NT	NT	NT	NT
Crude *E. coli* P 5 (O group 141)	≥ 20	NT	80	NT
Crude *E. coli* B 2577/67 (O group 147) I	≥ 20	40	> 20	200
Crude *E. coli* B 2577/67 (O group 147) II	≥ 20	30	≥ 20	160
Crude *E. coli* 537/67 (O group 149)	NT	NT	NT	NT
Crude *E. coli* 853/67 (O group 149)	40	300	> 20	650

* Tested with approximately 2 minimal effective doses of the enterotoxins.
† Tested with approximately 3 blueing doses of enterotoxin.
§ Titres in reciprocal values.
$ NT not tested.

RESULTS

Both the isolated and the crude *V. cholerae* exo enterotoxins and five of the seven crude *E. coli* enterotoxins caused pronounced fluid accumulation in the ileal loop assays (Table 1). This was assessed in at least two concurrently tested animals and reproduced one or more times. With some of the toxins the minimal effective dose (MED) was determined (Table 1).

Extensive studies were done to test whether *E. coli* enterotoxins, as has been established for the *V. cholerae enterotoxin*, give an increase in skin capillary permeability. This biological effect was demonstrated in several animals on two or more separate occasions for all of the enterotoxin preparations. This also included the two preparations which did not cause fluid accumulation in the gut tests. The individual variations among the rabbits

were more pronounced than those to occur if cholera toxin was used but still seemed associated with the responsiveness to the latter toxin, in the sense that rabbits in which one blueing dose of cholera toxin was 200 pg, or as occasionally seen even 100 pg, were the most sensitive also to *E coli* enterotoxin Rabbits requiring 1–5 ng of cholera toxin for skin blueing reactions were sensitive only to the enterotoxin preparations of the *E coli* strains 853/67 and B2577/67 These toxin preparations were also those with the highest titre of skin active material as determined with serial dilutions of the various preparations in sensitive animals (Table 1) Heating at 65° C for 10 minutes destroyed the skin activity of all the *E coli* preparations, except of undiluted 853/67 toxin where skin activity was still demonstrated whereas a heated 1 10 dilution was inactive

Neutralization studies in the ileal loop test revealed that antiserum to isolated exo-enterotoxin from *V cholerae* neutralized the diarrhoeogenic effect of crude and isolated cholera toxin at a similar titre (Table 2) This antiserum also neutralized the enterotoxins from *E coli* of various O groups as tested in two animals and obtaining identical results (Table 2) With the toxin from *E coli* strain 915/66 the neutralization by cholera antitoxin tested at a 1/20 dilution was questionable, in both animals the toxin serum mixture gave approximately half the fluid accumulation of that caused by the toxin TG mixture (mean 1 4 ml/cm compared to 2 9 ml/cm) The neutralizing capacity of the anti cholera toxin was titrated with the enterotoxin from *E coli* 853/67 The titre was approximately 1/10 of that noted for this serum if cholera toxin was used (Table 2)

Cross reactivity was also observed between the enterotoxins of various *E coli* strains and the cholera exo enterotoxin in ileal loop neutralization tests with antiserum to partially purified enterotoxin from *E coli* P 5 This antiserum showed a neutralization titre of 1/80 against the homologous toxin n l also neutralized the other *E coli* enter toxins except that of *E coli* 915/66 where the effect was questionable This serum had effect also on isolated cholera exo enterotoxin, but using this toxin, the antiserum titre was only 1/5

Cross neutralization studies using the intra dermal assay were also performed The amount of enterotoxin tested after incubation with antiserum dilutions was approximated to 3 blueing doses (BD) It was found that the antiserum to cholera toxin neutralized *E coli* toxins, but at lower titres than against the cholera toxin (Table 2) The antiserum to the enterotoxin of *E coli* P5 neutralized the enterotoxins produced by *E coli* of other O groups but at varying titres This antiserum also neutralized the skin activity of cholera exo enterotoxin but at a lower titre than *against any of the tested E coli enterotoxins*

DISCUSSION

This study shows that porcine enteropathogenic *E coli* bacteria of different serogroups produced heat-labile enterotoxins which shared antigenic determinants Thus an anti serum to the enterotoxin of one *E coli* strain could neutralize enterotoxins produced by other strains, although at a different titre Also rabbit antiserum to isolated *V cholerae* toxin neutralized the crude *E coli* enterotoxins Similarly, the isolated *V cholerae* toxin was neutralized by anti *E coli* toxin, these cross neutralization titres however, were considerably lower than those against the "homologous" enterotoxin The findings reveal that *E coli* and *V cholerae* produce enterotoxins which are immunologically cross reactive but not identical Similar conclusions were reached by *Smith & Sack* (1973) in a recent study of enterotoxins from *E coli* isolated from man It remains to be tested whether the antigenic similarities of the enterotoxins produced by *V cholerae* and various *E coli* strains could be utilized for induction of protective cross immunity

Knowledge about the properties of enterotoxins produced by *E coli* is limited by the fact that such enterotoxins have not yet been obtained in a purified form As in the case of

the cholera toxin the diarrhoeogenic effect seems to be mediated through the activation of intestinal cell adenyl cyclase (*Kimberg et al* 1971, *Sharp* & *Hynie* 1971, *Pierce* 1973), but the toxins from the two bacterial species differ with respect to the kinetics in the diarrhoeogenic action (*Pierce* 1973, *Carpenter et al* 1969) Another difference that has been reported is the inability of the *E coli* enterotoxins to induce increased capillary permeability demonstrable in the rabbit intradermal test In the present study, we show that this difference seems to be quantitative rather than absolute, since rabbits with high sensitivity to the permeability factor activity of isolated cholera toxin were also sensitive to a similar activity in all of the *E coli* enterotoxin preparations This activity was not demonstrable in preparations which had been heated at 65° C for 10 minutes, a procedure reported to destroy the heat labile but not the heat stable type of *E coli* enterotoxin (*Gyles* 1971) The skin activity was also neutralized by antiserum to a partially purified *E coli* enterotoxin and by antiserum to isolated cholera toxin, which indicates that the skin active material is antigenic as well as heat labile Most likely it consists of the heat labile toxin which is also active in ileal loops It may be expected that bio assaying of enterotoxins of *E coli* by the intradermal test will facilitate the efforts to purify the toxins

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SENSITIVITY TO NORMAL BRAIN ANTIGENS OF BLOOD LYMPHOCYTES FROM PATIENTS WITH GLIOMAS

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Immunological sensitivity to normal brain antigens of blood lymphocytes from neurosurgical patients and healthy subjects was investigated *in vitro* Lymphocytes from 9 out of 14 patients with gliomas of varying histological malignancy were stimulated into enhanced DNA synthesis by two soluble extracts of brain tissue Lymphocytes from one patient with brain infarction were also slightly stimulated The mechanisms by which such immunity may be induced are discussed

The central nervous system (CNS) is immunologically separated from the rest of the organism by its lack of a lymphatic drainage system Thus, antigens present only in the CNS, and not introduced to the lymphatic system elsewhere, remain unnoticed and do not induce immunity (14) When CNS material, whether isologous, homologous or heterologous, is injected into a test animal, an immunological response that leads to encephalomyelitis is aroused (1) In man, malignant neoplasms or tissue-damaging processes in the CNS may cause leakage of tissue-specific antigenic material, presenting it to the lymphatic system in an immunogenic way

When lymphocytes pre sensitized to a certain antigen are cultured in the presence of the antigen *in vitro*, their DNA synthesis is enhanced and they are transformed into lymphoblasts as a result of the immunological recognition of the antigen (13)

In the present study peripheral blood lymphocytes from patients with gliomas and other neurosurgical diseases were tested for sensitivity to two extracts of normal human CNS tissue

MATERIALS AND METHODS

Tissue extracts Extracts were prepared from the brain and liver of a 24-year old male traffic accident victim in the following way Twelve hours after death 150 g of the white matter of the brain and 150 g liver tissue for a control extract were collected and homogenized with an Ultra-Turrax homogenizer (Janke & Kunker Kg, Staufen, W Germany) in twice the volume of acetone The homogenates were thereafter washed five times in twice the volume of ethyl ether acetone (1 1), and dried on blotting paper at room temperature, suspended in 400 ml Sorensen s 0 1 M sodium citrate buffer (pH 4 3), and stored at +4° C overnight The following morning they were centrifuged at 4500 × *g* for one hour, and the supernatants dialyzed against distilled water for 24 hours After centrifugation at 4500 × *g* for one hour the supernatants were lyophilized

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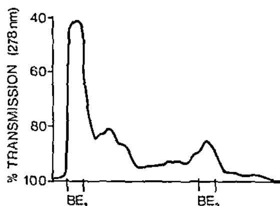


Fig 1 Chromatographic pattern of citrate buffer extract of defatted brain homogenate on a carboxy methyl cellulose column The two fractions, BE_1 and BE_2, used in the experiments, are indicated on the abscissa

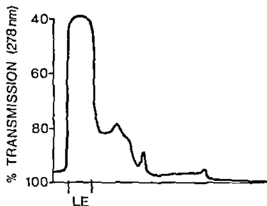


Fig 2 Chromatographic pattern of citrate buffer extract of defatted liver homogenate on a carboxy methyl cellulose column Fraction LE as depicted on the abscissa was used in control cultures

wavelength 278 nm in a Hitachi Perkin Elmer spectrofotometer The peak fractions depicted in Figures 1 and 2 were desalted on a coarse Sephadex G 25 column equilibrated with 0 01 N NaOH, and lyophilized The brain extracts were designated BE_1 and BE_2 as defined by Figure 1, and the liver extract was designated LE (Fig 2)

Each of the extracts was tested for encephalitogenic activity by injecting 150 μg suspended in 0 4 ml of distilled water and emulsified in 0 6 ml of Freund s complete adjuvant into the footpads and under the skin of three guinea pigs

Stock solutions used in the cultures were made by dissolving 100 mg of each extract in 100 ml of PBS A tuberculin PPD stock solution containing 250 TU/ml of PBS was prepared (Batch RT 23, State Serum Institute, Denmark) for the control cultures

Lymphocyte cultures Thirty ml of venous blood was drawn into a heparinized syringe, and a cell suspension containing approximately 80 per cent lymphocytes was prepared by the Ficoll Isopaque density sedimentation method (3) The cells were counted using the dye exclusion test (trypan blue) and the cell concentration was adjucted to 1 5 × 10 viable cells in 1 ml of RPMI medium

Two ml of the cell suspension (3 × 10^6 cells) with 0 5 ml of either autologous serum (AS) or serum from a healthy blood group matched adult (CS), were cultured in 35 mm Falcon Petri dishes (Falcon Plastics, Los Angeles Calif, USA) To each culture 0 2 ml of either one of the stock solutions were added and the cultures were incubated for 7 days at +37° C in a humidified atmosphere with 5 per cent CO_2 in air At the end of the incubation period a 60 minute tritiated thymidine pulse was given to the cultures by add ng 0 1 ml of a stock solution with an activity of 25 μCi/ml The culture dishes were then placed on an aluminium tray precooled to −20° C to stop the DNA synthesis immediately From each culture 1 0 ml was transferred to Wassermann tubes and placed in −20° C for later determination of radioactivity incorporated in the cells undergoing DNA synthesis Cell preparations were made from the rest of the cultures in a cytocentrifuge (Shandon Scientific Co, London N W 10, England) and stained with May Grunwald Giemsa for detection of possible bacterial or fungal infections in the culture and for morphological study of the cells

The samples for liquid scintillation counting were processed as follows The suspensions were thawed and after adding 5 ml of PBS, they were centrifuged at 2000 × g and +4° C for 10 minutes The supernatant was discarded, one drop of bovine serum was added, and after mixing, 5 ml of TCA in water were added The mixed material was centrifuged at +4° C and 2000 × g for 10 minutes The supernatant was discarded and 0 5 ml of 2N NH_3 were added After thorough mixing, 0 2 ml of this suspension were spread on a filter pad (Whatman glass fiber paper GF/A) in a scintillation vial and dried at 110° C for one hour Five ml of scintillation liquid were added and the radioactivity determined in a Packard scintillation counter

RESULTS

Encephalitogenic activity of the extracts BE_1 caused encephalitis as demostrated by hind leg paralysis in all three guinea pigs, 25, 26, and 28 days after immunization Perivascular infiltration of lymphocytes and plasma cells was seen in the CNS on histological examination The guinea pigs that received BE_1 lived longer (8, 19 and 20 weeks), and died with

TABLE 1 *CPM in Samples from Different Cultures (For Explanation of Abbreviations See Text)*

Patient	Age and sex	Diagnosis	Days before (B) or after (A) surgery	LE		BE_1		BE_2		PPD	
				AS	CS	AS	CS	AS	CS	AS	CS
Group I											
1) KM	52 F	Astrocytoma I	1 B	19	71	23	139	57	128	2156	2251
2) MS	23 M	Astrocytoma IV	6 A	41	61	64	212	36	102	1283	2597
3) AH	67 F	Astrocytoma I	1 B	19	24	130	177	149	157	3349	4608
4) SH	30 F	Astrocytoma IV	10 A	29	33	120	264	79	204	1491	1347
5) KH	50 M	Astrocytoma IV	2 A	33	59	161	104	121	169	2814	2524
6) HH	57 F	Astrocytoma IV	3 B	29	43	244	280	355	105	2019	2116
7) AK	22 F	Ependymoma	10 A	23	31	115	153	143	167	2599	2188
8) VK	36 M	Astrocytoma III	4 A	33	44	228	202	208	184	2045	2052
9) BS	61 F	Ependymoma	21 A	66	50	291	155	103	202	2088	1516
Group II											
10) EK	37 M	Astrocytoma III	6 B	30	41	31	28	32	31	2152	2271
11) TK	8 F	Spongioblastoma	3 B	52	68	74	69	27	26	2941	2773
12) MP	15 F	Spongioblastoma	4 B	23	30	19	34	51	57	2624	2638
MP	15 F	Spongioblastoma	11 A	32	19	15	33	21	28	3061	2208
13) KM	57 F	Astrocytoma I	12 A	37	23	41	32	35	29	133	246
14) HM	64 F	Astrocytoma III	48 A	22	42	56	40	22	29	3381	2874
Group III											
15) MK	55 F	Brain infarction	–	57	61	79	58	87	39	3938	2947
16) PM	44 M	Metast ca	7 A	47	44	61	39	45	73	4018	4721
17) TM	56 M	Metast ca	21 A	67	49	39	60	66	19	1735	1803
18) UR	64 M	Metast ca	5 A	46	44	45	36	31	48	914	731
Group IV											
19) TW	30 M		–	44	39	44	53	26	52	2958	2801
20) RP	30 F	–	–	11	28	28	20	26	16	3296	1172
21) CS	22 F		–	23	22	32	16	33	21	1856	1923
22) MS	30 F		–	21	14	20	56	17	70	1520	1009
23) AT	35 F	–	–	41	69	25	50	41	47	3688	2108
24) AB	37 F		–	40	51	68	59	57	29	1786	3107
25) ML	30 F	–	–	42	33	38	24	29	33	1117	1520
SV	36 F		–	48	37	42	36	32	58	133	163
ST	21 F	–	–	40	29	33	35	31	42	2148	1812

clinical signs of encephalitis (hind leg paralysis, cachexia and apathy) Histologically, however, no signs of either inflammation or demyelinization could be detected LE did not cause any changes in the appearance or behaviour of the guinea pigs within 6 months, and when the animals were killed after that time, no histopathological changes were seen in the CNS

Lymphocyte stimulation The results from the lymphocyte stimulation experiments are summarized in Table 1 The mean of the counts per minute (CPM) in the samples from all cultures with autologous serum and LE is 37.8 ± 14.2 (SD) and of those with control serum 42.0 ± 16.4 The brain extracts were considered to have stimulated the DNA synthesis in the cases where the CPM exceeded the above means plus 2 6 times the standard deviation (80 7 in cultures with autologous serum, and 84 6 with homologous serum), which is the 99 per cent confidence limit, and thus taken to reflect an immunological recognition of antigens in the extracts

On this basis the patients tested were divided into three groups in Table 1 Group I comprises those glioma patients whose lymphocytes were stimulated by the brain extracts In patients 1 and 2 there was no stimulation of the DNA synthesis when the cells were cultured in autologous serum Whether this was due to specific blocking activity of the sera (9) interfering with the specific immunologic reaction of the cells with their antigens remains to be established

Group II includes those glioma patients whose lymphocytes were not stimulated by the brain extracts No difference in the clinical course of the disease as to severity or duration of symptoms in the two groups could be found, nor was there any clear difference between histologically malignant and benign tumours or their size Neither was there any difference between these two groups with regard to whether the test was done before or after surgery In accordance with this similar results were obtained with lymphocytes from patient 12 tested both four days before and eleven days after the operation

Group III consists of patients with brain lesions other than gliomas Patient 15 had an infarction in the right temporal lobe According to the criteria given above her lymphocytes were slightly stimulated by BE_2 in the culture with autologous serum

Finally, Group IV is composed of nine healthy laboratory personnel

All persons tested had received a Calmette vaccination in childhood No Mantoux tests were performed to investigate whether the low grade of stimulation in some cases correlated with unresponsiveness in skin tests None of the patients was given cytostatic or irradiation therapy at the time when the blood samples were drawn

DISCUSSION

The lymphocyte blastogenic response to different antigens *in vitro* has been studied extensively (13) Non sensitized lymphocytes have been shown to undergo blast transformation when cultured together with living homologous and heterologous non neoplastic cells (6, 13, 10) Tumour immunity has been assayed employing such methods by using both living tumour cells (4, 5, 19 17, 2), and subcellular tumour preparations (15, 8) A blastogenic response of autologous lymphocytes to living tumour cells *in vitro* may be a summation of primary and secondary stimulation (5, 18)

Soluble antigens, on the other hand are reportedly able to stimulate only presensitized lymphocytes *in vitro* (12, 8) In the present experiments lymphocytes from only such subjects were stimulated, who probably had been previously exposed to CNS specific antigens through disseminating cells from glial tumours or CNS tissue resorbed after damage

Unexpectedly, both of the brain extracts that were prepared essentially as described by *Kibler & Shapira* (11) were encephalitogenic when injected into guinea pigs though there was a distinct difference of time after which the symptoms appeared These workers isolated an encephalitogenic protein from a fraction corresponding to BE It was therefore anticipated that only BE should cause en

hancement of the DNA synthesis in the lymphocyte cultures

Cellular immunity to normal brain antigens in glioma patients has previously been studied by other methods Thus, normal CNS tissue *material causes delayed type hypersensitivity* in some patients with malignant gliomas (7), and peripheral blood lymphocytes from such patients are cytotoxic to normal glial cells *in vitro* (20)

The mechanism by which the sensitization to normal CNS antigens is induced in glioma patients is not clear It may be a sequel of disseminating cells from the tumour introducing glia-specific antigens not earlier confronted by the immune system, or it might be the outcome of resorption of damaged CNS tissue Tumour cells have been found in the peripheral blood of glioma patients (16), but still metastases outside the CNS are extremely rare in these patients This may be due to a *strong immune response caused not only by* tumour-specific antigens but also by normal glia specific antigens Glioma patients generally do not show signs of encephalitis This may be due to protective factors in their sera (9)

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GLIA-SPECIFIC ANTIGENS IN CELL CULTURES FROM RABBIT BRAIN, HUMAN FOETAL AND ADULT BRAIN, AND GLIOMAS

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Glia specific antigens were demonstrated in cells cultured from human foetal and adult brain tissue, baby rabbit brain tissue, and established cell lines from human malignant gliomas by the indirect immunofluorescent staining technique The significance of such antigens in demyelinating processes is discussed

Malignant gliomas and normal brain tissue share antigens that are not found in other tissues (11) It has also been shown that some glioma patients are sensitized to extracts of normal brain tissue in skin tests (4) and when the blastogenic response of their lymphocytes is tested in vitro (12) Moreover, peripheral blood lymphocytes from glioma patients are cytotoxic to both adult and foetal glial cells in culture (13)

These facts prompted us to investigate whether such tissue specific antigens could be demonstrated in cell cultures from normal and neoplastic brain by heteroimmune sera and indirect immunofluorescence

MATERIALS AND METHODS

Anti brain sera Normal brain tissue from a 28 year old male traffic accident victim autopsied within twelve hours from death was homogenized in distilled water at +4° C with an Ultra Turrax mincer (Janke & Kunker Kg Staufen, West Germany) and a tube roller homogenizer in an ice bath The homogenate was lyophilized and subsequently divided into 10 mg aliquots in tightly sealed glass tubes and stored at room temperature

Two rabbits were immunized by weekly subcutaneous injections of 10 mg of lyophilized human brain dissolved in 0 5 ml of distilled water and emulsified in 0 5 ml of Freund's complete adjuvant (Difco Lab, Detroit, Mich, USA) The injections were given alternately in the left scapular, right gluteal, right scapular and left gluteal regions. Serum was collected from both rabbits prior to and during immunization by monthly bleedings

After immunization for four months the rabbits developed severe signs of encephalomyelitis (paraplegia, cachexia, apathy) They were therefore sacrificed and blood was drawn by heart puncture

Immunodiffusion assay and absorption of sera The sera were tested against lyophilized human brain, lung, liver, kidney, placenta and glioblastoma tissue and pooled serum, as well as rabbit brain and lung by double diffusion in 1 per cent agar gel in PBS ('Ionagar No 2, Oxoid Ltd, London England) To absorb antibodies reacting with antigens present also in other tissues than brain, 50 mg of each of lyophilized human placenta liver lung and kidney, and 30 mg of lyophilized pooled human serum were moistened with distilled water and thoroughly mixed with 1 ml of immune serum This was incubated at +4° C for 24 hours and

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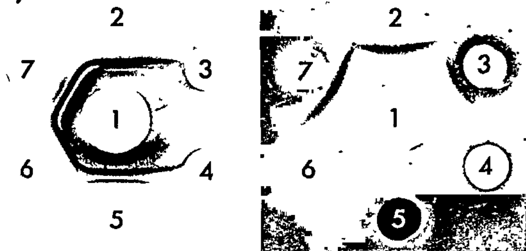


Fig 1 and 2 Double diffusion precipitin reactions of unabsorbed (well 1 in Fig 1) and absorbed (well 1 in Fig 2) anti brain serum against human brain (well 2), rabbit brain (well 3), rabbit lung (well 4), human lung (well 5), human liver (well 6), and human glioblastoma multiforme (well 7)

thereafter centrifuged at 4000 × *g* for 10 minutes

To complete the absorption, the sera were further incubated at + 4° C for 24 hours with cultured normal human fibroblasts removed from culture with a rubber policeman at a ratio of 6–9 × 10^6 cells per ml of serum, and again centrifuged as above

Cell cultures Foetal tissues were obtained from therapeutical abortions Only foetuses with a crown to-heel length exceeding 12 cm were used Normal adult brain tissue and skin were taken at neurosurgical operations Rabbit tissues were cultured from a 4 day-old baby rabbit

with scissors and suspending them in minimum essential medium (Flow Laboratories Irvine Scotland) supplemented with 10 per cent bovine serum, glutamine and amino acids After the cells had grown out to confluent monolayers they were detached by trypsinization (0 25 per cent trypsin in PBS, Orion Oy, Helsinki Finland) and seeded out on cover glasses in 50 mm diameter Petri dishes (Falcon Plastics, Los Angeles, Calif USA) After two or three days the cover glasses were washed in PBS dried in a standing position on blotting paper at room temperature, and fixed with acetone for 20 min at 20° C

In addition to the newly started cultures established cell lines from fifteen gliomas one synovial sarcoma and one osteosarcoma, obtained through the courtesy of Dr *Bengt Westermark* (Wallenberg Laboratory, Uppsala, Sweden) were tested Cover glass monolayers from these were prepared as above

Immunofluorescence assay After absorption of the sera, their anti glia antibodies were assayed by indirect immunofluorescence employing a fluorescein isothiocyanate labelled sheep anti rabbit IgG serum with a molar F/P ratio of 2 4 (FAR) (7)

The cover glasses with the cultured cells were dipped in PBS, placed in a moist chamber, and covered for one hour at room temperature with absorbed immune serum or similarly absorbed control serum in dilutions between 1 8 and 1 16 The glasses were then washed with PBS for 45 minutes and covered with FAR containing 20 μg of protein per ml for 30 minutes The washing was repeated

RESULTS

Double diffusion tests of the immune sera In Figures 1 and 2 the reactions are shown of one of the immune sera collected after immunization for four months when the rabbit suffered from severe encephalomyelitis It is seen that after absorption with non-nervous tissues as described above, one precipitin line

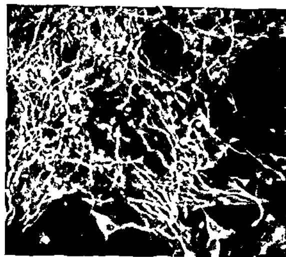


Fig 3 A cluster of oligodendrocytes on astrocytic cells cultured from human foetal brain Passage 3 in vitro Original magnification 100×



Fig 4 Astrocytic cells among non glial' cells cultured from human foetal brain Passage 12 in vitro Original magnification 200×

was left against normal human brain, human glioblastoma and rabbit brain Further absorption with 100 mg of lyophilized brain tissue per ml of serum removed this reaction

The pre-immunization sera did not react in double diffusion before nor after absorption

Morphology of the cell cultures In the cultures started from human foetal brain, two morphologically distinct "glia like" cell types were seen in May Grunwald-Giemsa staining One consisted of polygonal type cells with a relatively large round or oval pale staining nucleus containing one or several nucleoli This type had an abundant cytoplasm and a delicate network of fibrils close to the nucleus They also had plasmatic prolongations, some of which were far-stretching and thin, some broad and blunt This type of cell was called astroglia

The other glia-like cell type in the human foetal brain cultures had a small round compact dark-staining nucleus surrounded by a narrow rim of cytoplasm Two or more straight slender processes reached out in different directions These cells seemed unable to grow directly on the glass since they always appeared as clusters of varying size on top of the astroglia They were attached only at the nucleus and the ends of the tiny cytoplasmic prolongations, as seen by the wavy movements of these with the growth medium when the cultures were examined in a microscope Their number decreased quickly when the cultures were propagated through several passages so that in passages over ten they were hardly ever encountered These cells were called oligodendroglia

In the foetal brain cultures there were also cells that did not have the "glia-like" features described above These cells, too, had an abundant cytoplasm, but no cytoplasmic processes or perinuclear network was seen in them They were therefore designated as non-glial cells Fibroblasts were not seen in these cultures

When the foetal brain tissue was put in culture, the meninges were torn off and cultured separately From these an epithelioid cell type grew out These cells also had a polygonal shape with some short blunt processes and a nucleus resembling that of the astroglia They were called meningeal cells

On top of the meningeal cells, clusters of

oligodendroglia occured, similar to those in the brain cultures These apparently origin ated from fragments of brain tissue torn off with the meninges

In the cultures from baby rabbit brain the majority of the cells were similar to the hu man foetal non glial cells in that they were flake shaped and had no cytoplasmic pro cesses On top of the monolayer formed by these cells several glia like cells appeared in clusters or as single cells Their morphologi cal features ranged from gemistocytic astro cytes with abundant granular cytoplasm th ck irregular processes and large pale stain ing nuclei through fibrillary astrocytes with scantier cytoplasm and thinner processes to fusiform bipolar spongioblastic cells

In the skin cultures ordinary fibroblasts were seen For morphological description of human adult brain cell cultures and the estab l shed glioma cell lines see *Ponten & Macin tyre* (1968) and *Westermark et al* (1973)

Immunofluorescent staining In the human foetal brain cultures the oligodendroglia ex hibited a strong fluorescence clearly visible in the most delicate cytoplasmic prolonga tions (Fig 3 and 4) On examination at a high magnification the fluorescence appeared as a dense fibrillary network This was con fined to the cellular membrane but there was a distinct fluorescence situated in the perinuclear cytoplasm as well In the astro glial cells a looser network was seen but the fluorescence was not as intense as in the oli godendroglia

When the cultures were propagated through ten or more passages in vitro the oligodendroglial cells disappeared and the only cells with positive immunofluorescent stain ng were the astroglial cells Examples of immunofluorescence in newly started and later passages of human foetal brain cell cul tures are shown in Figures 3 and 4

In the human adult brain cell cultures the astrogl al cells had a perinuclear fluorescence that was considerably weaker than in the foe tal cells and did not reveal any structural characteristics (Fig 5) Occasional bipolar spongioblast like cells on top of the astroglia

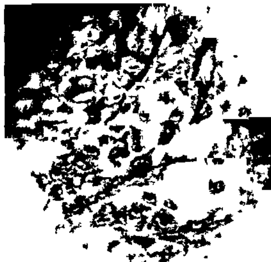


Fig 5 Human adult glial cells Passage 7 in vitro Original magn ficat on 100×

monolayer showed a more intense fluores cence

The fluorescence in the established glioma cell lines was clearly bound to the cytoplasm In some of the lines the fluorescence revealed granular cytoplasmic structures but in most of them it was rather amorphous as seen in Figure 6 The numerous cells stained in m to



F g 6 An establ shed cell l blastoma multiforme P magnif cat on 200×



Fig 7 One fibrillary astrocyte in the middle and one gemistocytic astrocyte to the left in a culture from baby rabbit brain Passage 6 in vitro Original magnification 200 ×



Fig 8 Gemistocytic astrocytes on "non glial cells in a culture from baby rabbit brain Passage 11 in vitro Original magnification 100 ×

sis had the most intense fluorescence in these cultures, apparently due to concentration of the antigens

The glia like cells in the cultures from baby rabbit brain had a very distinct membrane fluorescence that revealed a similar fibrillary network to that seen in the human foetal astroglial cells This glia-specific fluorescent staining is demonstrated conclusively in Figures 7 and 8

The flake-shaped "non glial" cells were consistently negative The skin fibroblasts and the cells of the two sarcoma lines were not stained by the immune sera The pre immunization sera from the rabbits did not stain any cells

Absorption of the sera with 100 mg of lyo philized normal human brain removed all the fluorescence

DISCUSSION

The experimental allergic encephalitis (EAE) has served as a model for the study of autoimmune diseases for half a century This pathological condition is brought about in laboratory animals by immunization with isologous, homologous or heterologous CNS material, which results in perivascular and diffuse infiltration with inflammatory cells, demyelination and subsequent gliosis of the CNS (10)

The location of the antigens in the CNS that elicit the immune reactions in EAE has not been fully clarified The myelin associated basic proteins common to different species are known to be potent inducers of the disease (1), but as yet it has not been solved in what way these proteins are associated with the myelin

It is, however, known that both lymphocytes and sera from animals with EAE exert lytic effects on glial cells in culture, a process that probably is based on antigens on the cell surface (6, 2)

The present study was undertaken in order to investigate whether glia-specific antigens can be demonstrated in cultured cells of glial origin that may be the basis for such reactions

Rabbits were immunized with lyophilized normal adult human brain tissue When the

animals developed severe EAE, their sera were collected and thoroughly absorbed with non-nervous human tissues until they only reacted in double diffusion tests with lyophilized human and rabbit brain tissue, and with human glioma tissue, but not with non-nervous human or rabbit tissues

In indirect immunoflurescent stainings with the fully absorbed anti-brain sera of cell cultures started from foetal and adult human brain, human gliomas, and baby rabbit brain, cells with "glial" morphology were specifically stained The specificity of the staining was demonstrated by the fact that cells with "non glial" morphology in the same cultures were not stained Moreover, appropriate controls in which cells cultured from other than nervous tissues were not stained by the absorbed immune sera, and the pre-immunozation sera did not stain cells of glial origin, offered strong evidence for the glia specificity of the immune sera after absorption

Finally, the fact that rabbits immunized against human brain produced antibodies that in the indirect immunofluorescent experiments specifically stained cultured rabbit glial cells, indicates that there are common glia specific antigens that mainly seem to be associated with the cellular membrane and that they normally are beyond the recognizing mechanisms of the lymphatic system

The cell chiefly involved in the formation of the CNS myelin sheaths is the oligodendrocyte (3) On the basis of the present findings it is a tempting thought that an important feature of EAE and possible in human demyelinating diseases as well, is an immunologic reaction against specific constituents of the glial cellular membrane perhaps in part identical with the myelin associated basic proteins

Cultured human and animal cells of nervous origin are known to retain their capability to produce organ specific substances (5, 8) In the present study we have shown that such cells also express their specific antigens even after prolonged culture

In earlier studies we have shown that blood lymphocytes from patients with gliomas are cytotoxic to glial cells in vitro (13), and that they are stimulated into enhanced DNA synthesis by encephalitogenic extracts of normal human CNS tissue (12) What bearing these findings have on any human disease involving the CNS cannot as yet be concluded It is however, possible that the normal glia specific antigens, in addition to tumour-specific antigens, may play a role in preventing the formation of distant metastases from gliomas, that are only rarely seen

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RAISED LEVELS OF IMMUNOGLOBULINS IN SERUM OF HYPERTENSIVE PATIENTS

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In a series of 84 hypertensive patients, 50 men and 34 women, the levels of serum immunoglobulins were examined. Levels of the immunoglobulins were found to be increased in about 30 per cent of the patients. The study supports the view that immunological factors are involved in at least some cases of essential hypertension. The antigens against which the immunoglobulins are directed have not yet been identified.

Immunological factors seem to be involved in essential arterial hypertension in man. These factors include both a hypersensitivity of the delayed type (*Olsen* & *Loft* 1973a, 1973b) and of the humoral type (*Ebringer* & *Doyle* 1970, Olsen 1972).

The aim of the present work has been an examination of the frequency at which the immunoglobulins are increased in the serum of essentially hypertensive patients and whether it is possible in a large series to observe a difference in the severity of hypertensive disease in patients with raised levels of serum immunoglobulins and in those with normal levels.

MATERIAL AND METHODS

In 84 hypertensive patients suffering from primary hypertension, the immunoglobulins in the serum were analysed. Patients with arterial hypertension and elevated levels of immunoglobulins who were suspected of liver disease or collagen disease, were not included in the series. The series of patients comprised 50 men and 34 women. At the time of estimation of the immunoglobulins, 60 of the patients were in antihypertensive treatment with one or more of the following antihypertensive drugs: Hydrofluomethiazidum, Polythiazidum, Hydrochlorthiazidum, Methyldopa, Betanidini sulfas, Hydralazini chloridum, Propranolol. The other 24 patients were untreated. The blood pressure before antihypertensive treatment varied from 300/170 mm to 170/110 mm Hg, averaging about 225/130 mm Hg. The blood pressure at the time of measurement of the concentration of the serum immunoglobulins varied from 250/130mm Hg to 130/80 mm Hg, averaging about 165/110. The renal function was measured on the basis of the concentration of the serum creatinine. Eleven of the 50 men showed a variation of 1.5 mg/100 ml to 2.6 mg/100 ml, which is a slight increment of the concentration of serum creatinine. Ten of the 34 women showed also a slight increase in the serum creatinine with a variation of 1.3 mg/100 ml to 2.0 mg/100 ml.

The age of the patients varied from 72 years to 24 years.

The levels of serum immunoglobulins in hypertensive patients were compared with the reference values for IgG, IgA and IgM derived from *Weeke*

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* Analyse of serum immunoglobulins were carried out by Department of Clinical Chemistry, Bispebjerg Hospital, Copenhagen, Denmark.

TABLE 1 *The Absolute Levels of IgG, IgA and IgM in the Serum from the Fourteen Hypertensive Men in whom Values of the Serum Immunoglobulins Were Increased*

Age (years)	IgG (g/l)	IgA (g/l)	IgM (g/l)	BT (mm Hg) untreated	BT (mm Hg) treated	Antihypertensive drug	Serum creatinine
29	16 0	1 5	1 90	170/120	140/110	6)	1 1
35	14 5	3 8	0 40	160/100		–	1 1
59	15 0	4 3	–	220/120	180/120	1),3),4)	1 2
45	14 5	4 1	0 62	220/140	160/100	1),3),4),5),6)	1 4
37	18 5	2 3	0 80	220/140	140/90	1),5),6)	1 1
57	17 0	6 5	1 40	170/120	160/100	6)	1 2
31	13 0	4 4	0 48	150/110	115/80	6)	1 0
68	19 0	4 8	0 68	200/120	185/105	2) 3)	1 5
72	12 0	3 7	0 35	200/130	210/110	2),3)	2 6
67	13 5	3 8	0 92	230/150	160/90	2)	1 5
53	19 9	3 2	0 58	190/120	130/80	6)	1 4
41	17 0	2 1	0 39	180/140	140/115	1) 3),4)	1 6
43	14 0	3 8	1 20	190/110		–	0 9
61	17 5	3 1	0 20	210/130		–	1 4
Reference values	7 0–16 0	0 5 3 5	0 2 1 50				0 9 1 4

1) Hydrochlorthiazidum
2) Hydroflumethiazidum
3) Methyldopa
4) Betanidini Sulfas
5) Hydralazini Chloridum
6) Propranolol
7) Polythiazidum

TABLE 2 *The Absolute Levels of IgG, IgA and IgM in the Serum from the Eleven Hypertensive Women in whom Values of the Serum Immunoglobulins Were Increased*

Age (years)	IgG (g/l)	IgA (g/l)	IgM (g/l)	BT (mm Hg) untreated	BT (mm Hg) treated	Antihypertensive drug	Serum creatinine (mg/100 ml)
33	14 5	4 3	0 40	190/120	130/100	6)	0 9
30	16 0	4 1	1 32	215/135	140/100	1) 6)	0 8
56	22 5	3 8	0 82	200/110	180/90	1) 3)	1 7
51	20 0	2 4	0 60	220/140	150/90	7)	1 3
64	11 0	3 8	0 58	270/150	250/130	1),3)	2 0
70	16 5	5 4	0 60	235/120	155/85	1)	0 7
60	20 0	4 1	0 80	180/130	155/85	1) 3)	1 0
63	11 5	5 0	0 90	200/115	160/95	1),3)	1 4
50	19 5	6 3	0 74	300/170	160/100	1) 3) 5)	1 5
66	20 0	4 1	0 42	230/135	175/115	2)	0 7
33	19 0	2 4	0 90	175/110		–	0 8
Reference values	7 0 16 0	0 [illegible] 3 5	0 2 1 50				0 8–1 2

1) Hydrochlorthiazidum
2) Hydroflumethiazidum
3) Methyldopa
4) Betanidini Sulfas
5) Hydralazini Chloridum
6) Propranolol
7 Polythiazidum

The accuracy of the three analyses was based on standards of purified IgG, IgA and IgM prepared in the laboratory Also the specific antibodies were prepared in the laboratory Precision expressed as coefficient of variation was from 5 to 7 per cent

RESULTS

Table 1 shows both the number of hypertensive men in whom the immunoglobulins, IgG, IgA and IgM were found to be increased and the absolute levels of the immunoglobulins in these patients compared with the normal range Table 2 shows the same data on hypertensive women

TABLE 3 *The Absolute Levels of IgG, IgA and IgM in the Serum from the Hypertensive Men in whom Levels of Serum Immunoglobulins Were not Increased*

Patients	IgG (g/l)	IgA (g/l)	IgM (g/l)
LT	12 5	2 4	0 70
CC	11 5	2 2	1 30
PJ	7 5	1 3	0 70
PS	9 0	2 1	0 70
AR.	15 0	1 5	0 40
P.M	11 5	2 0	1 00
JA	9 0	2 0	1 50
ED	11 0	1 7	1 30
OE	12 0	2 5	0 82
CH	14 0	2 9	0 43
FL.	15 0	2 9	0 52
K.P	12 5	1 9	0 32
LW	13 0	2 0	0 78
KW	15 5	2 6	0 99
AV	14 5	3 3	0 68
JMH	11 5	2 5	1 24
PM	13 0	1 7	0 56
ON	11 5	2 2	0 30
IJ	10 0	2 7	0 52
PJ	12 0	3 2	0 43
HH	8 0	1 9	1 49
JH	16 0	3 5	0 45
HH	9 5	2 2	0 70
HH	10 0	2 3	0 54
JF	14 5	2 4	0 40
GF	12 0	1 7	0 64
VD	16 0	2 3	0 44
OH	13 0	1 1	0 44
MG	11 0	1 8	0 76
CJ	15 0	3 2	0 35
H.H	13 0	1 9	0 60
VS	14 5	3 5	0 34
HH	14 0	1 7	0 50
OV	13 0	1 5	0 20
LH	9 5	2 3	0 87
PP	7 5	1 2	1 14
Reference values	7 0–16 0	0 5–3 5	0 2–1 50

TABLE 4 *The Absolute Levels of IgG, IgA and IgM in the Serum from the Hypertensive Women in whom Levels of Serum Immunoglobulins Were not Increased*

Patients	IgG (g/l)	IgA (g/l)	IgM (g/l)
GH	11 0	2 9	0 90
KN	13 0	2 1	1 50
KL	11 5	1 0	0 70
KT	9 5	1 4	0 90
IH	12 5	1 6	1 40
BP	10 5	2 0	0 70
GJ	13 5	2 5	0 46
ES	10 0	2 4	0 60
A.G	11 5	2 2	1 00
RE	7 0	1 1	0 50
KH	15 0	2 2	0 82
JK	13 0	1 5	1 28
GL	8 5	1 8	0 52
ML	13 0	2 2	0 60
MM	13 0	2 8	0 30
UP	15 5	1 6	1 45
VB	12 5	1 6	0 66
EB	12 0	3 0	0 30
EL	10 5	0 9	0 44
RP	8 5	2 3	1 21
EJ	13 0	2 9	0 50
RF	10 0	1 3	0 94
HM	16 0	2 2	0 58
Reference values	7 0–16 0	0 5 3 5	0 2 1 50

It is seen that levels of one or two of the three types of immunoglobulins were elevated in fourteen of the hypertensive men or in 28 per cent of these An increment of IgM was found only in one case Six of the hypertensive men with increased levels of serum immunoglobulins were untreated at the time of the examination

Concerning the hypertensive women, levels of one or two types of the immunoglobulins were elevated in eleven of these, or in 32 per cent An increment of IgM was not fou
Two of the hypertensive women ...

creased levels of serum immunoglobulins were untreated at the time of the examination

Thus, the increased levels of serum immunoglobulins in hypertensive patients, were not found to differ markedly in women and men

Table 3 and Table 4 show the levels of serum immunoglobulins in the hypertensive men and women in whom concentrations of the immunoglobulins were not increased

It applies both to men and women that there was no correlation between an elevation of the concentration of serum immunoglobulins and the severity of the arterial hypertension, the renal function or the type of the antihypertensive drug with which the patient was treated

DISCUSSION

The presented results demonstrate that levels of the immunoglobulins IgA and/or IgG were increased in about 30 per cent of the examined hypertensive men and women while an increment of IgM was found in only one case

So far, the role of these findings in the pathogenesis of the arterial hypertension or the hyptertensive vascular disease remains to be defined The antigens against which the immunoglobulins are directed are not known either

In this study, the majority of the hypertensive patients received antihypertensive drugs but no correlation could be drawn between the treatment and the raised levels of the serum immunoglobulins Furthermore an increment of immunoglobulins was observed in eight of the 24 patients who were not treated with antihypertensive drugs and the same was observed in a previously published report on a small series of hypertensive patients (*Olsen* 1972) Thus it is evident that the treatment does not influence the level of the immunoglobulins in the serum A difference in severity of the systemic hypertension in patients with increased levels of serum immunoglobulins and in patients in whom levels of the immunoglobulins were normal was not demonstrable by a comparison of the two groups

It seems likely that immunological factors of humoral type as well as of delayed type are involved in at least some cases of essential arterial hypertension (*Paronetto* 1965, *Ebringer* & *Doyle* 1970, *Olsen* 1972 *Olsen* & *Loft* 1973a, b), but only intensive studies in the future will give the answer to the question whether these factors are pathogenetic in the development of the hypertensive disease

However, the possibility exists that the elevated serum level of a certain immunoglobulin class does not necessarily indicate increased humoral hypersensitivity since it could just as well indicate a non specifically increased synthesis or a decreased catabolism

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DELAYED HYPERSENSITIVITY AND ARTERIOSCLEROSIS IN MAN

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By means of the leucocyte migration technique it is demonstrated that a hypersensitivity of the delayed type seems to be involved in at least some cases of arteriosclerosis in man The hypersensitivity is directed against unknown antigenic substances in the femoral arterial wall

It is likely that arteriosclerosis is the result of the reaction of the arterial wall to injury, regardless of the damaging factor (*Lorenzen* 1963) Many different factors have been mentioned in the literature as those responsible to the development of arteriosclerosis (*Lorenzen* 1963, *Kjeldsen* 1969) Among these, also an immunological mechanism has been mentioned (*Davies* 1969)

The aim of the present work has been to examine whether a delayed hypersensitivity directed against arterial antigens could be demonstrated in patients suffering from arteriosclerosis

METHOD AND MATERIAL

The method used was the leucocyte migration technique (*Søborg & Bendixen* 1967 *Bendixen & Søborg* 1969) A femoral artery without macroscopical atherosclerosis from a man who had been dead for about twelve hours was used for the production of arterial antigen(s) After a skin incision on the medial side of the thigh the femoral artery was removed under optimal aseptic conditions Fragments of the artery, including tunica intima media and adventitia were subsequently homogenized immediately by homogenizer pestles. The homogenization time was five minutes at 4° C Finally the homogenate was extracted in Hank s buffered salt solution over night at 4° C After centrifugation the supernatant was pipetted off from the sediment and the concentration of the protein in the supernatant was measured The migration of the leucocytes from arteriosclerotic patients and control persons was studied in culture chambers containing an arterial protein concentration of about 50 micrograms per ml The results were given as the migration index which is defined as the ratio between the migration area of antigen containing and antigen free cultures.

With a view to evaluating the specificity of the leucocyte migration technique in culture chambers containing femoral arterial proteins the migration was measured in culture chambers containing equal concentrations of protein from homogenized and extracted liver tissue derived from human subjects

Fourteen patients without arterial hypertension and suffering from arteriosclerosis were examined In seven of the patients the arteriosclerosis was localized mainly to the legs in four patients the arteriosclerosis was localized universally in one patient mainly to the coronary arteries and in two patients mainly to the aorta which was observed on the X ray of the thorax The age of the patients, ten men and four women, varied from 44 years to 78 years

Eleven persons without demonstrable signs of arteriosclerosis were used as control persons The age of the latter varied from 22 years to 59 years and the series comprised six men and five women

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RESULTS

The results of the leucocyte migration given as the migration index are seen in Fig 1 The

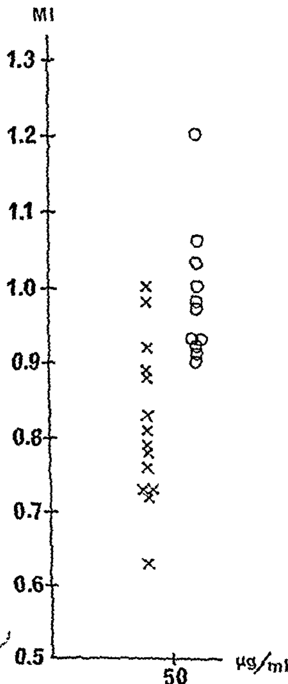


Fig 1 Arteriosclerotic patients (x) Persons without demonstrable signs of arteriosclerosis (o)

Ordinate Migration index (MI) *Abscissa* Micrograms per ml of *femoral* arterial protein in the culture chambers The figure *shows that* about 64 per cent of the *indices* from the arteriosclerotic *patients* are separated clearly from the *indices of* the persons *without demonstrable* signs of arterio sclerosis.

shows that the migration index of the arteriosclerotic patients varied from 0 63 to 1 00 Nine of the indices, or about 64 per cent of the fourteen indices from the arteriosclerotic patients, are separated clearly from the indices of the control persons in whom they vary from 0 90 to 1 2 The indices of the arteriosclerotic patients are significantly different from the indices of the control persons $P < 0.005$ (Wilcoxon Mann Whitney rank sum test)

In the cases in which a solution of protein from the liver was placed in the culture chambers in stead of protein from the femoral arterial wall the migration indices varied from 0 9 to 0 97

$P < 0.005$ if experiments using arterial protein in the culture chambers were compared with those using liver protein A comparison of the results obtained if the culture chambers contained liver protein and those of the control chambers did not reveal any significant difference between the two groups

DISCUSSION

The results show that a hypersensitivity of the delayed type may be involved in some cases of arteriosclerosis in man The role of this hypersensitivity in the pathogenesis of arteriosclerosis cannot be predicted for the time being

The problems concerning an immunological mechanism in the pathogenesis of arteriosclerosis have been reviewed in a paper by *Davies* (1969) In this paper it is concluded that exogenic allergens seem to be involved in some cases of arteriosclerosis while the possibility of an auto immune mechanism is not mentioned Studies by *Geertinger & Sorensen* (1970) and *Geertinger et al* (1970) supported the view that serum complement was an essential component in the development of experimental arteriosclerosis Whether complement had to be activated via an antigen antibody reaction could not be concluded but if serum complement is involved in the development of arteriosclerosis, this fact is in support of the immunological view

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EFFECT OF STANDARD BACTERIAL VACCINE ON INFLUENZA VIRUS INFECTION AND INTERFERON PRODUCTION IN GERMFREE MICE

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A standard bacterial vaccine (SBV) induced interferon (IF) production and reduced the mortality due to influenza A_2 virus infection in germfree mice These effects were quantitatively comparable in conventional and germfree mice The length of survival was somewhat shorter in conventional mice It is concluded, that SBV exerts a direct effect on the viral infection probably partly mediated by IF induction The role of resident bacterial flora is discussed

In previous communications we reported that a standard bacterial vaccine (SBV) reduced the mortality following influenza A_2 virus infection in mice (4) Furthermore, development of gross pulmonary lesions, growth of virus in the lungs and antibody titres against the virus were also reduced by intraperitoneally (i p) injected SBV, when the vaccine was given a few hours prior to viral infection Similar protection was obtained against para influenza 1 Sendai virus, Semliki forest virus (10, 17) and Vesicular stomatitis virus (4) We have suggested (4) as did *Singer & Hardegree* (17) that the protection may at least in part be mediated by production of interferon or interferon-like substance

It is well documented both in clinical and experimental studies, that viral respiratory infections are often complicated by secondary bacterial invasion, which may influence the outcome of disease (6, 14) Reduction or elimination of secondary invaders reduced the mortality and increased the length of survival in Sendai virus infected mice (3, 6) A possible effect of SBV on this secondary bacterial invasion should be considered We decided to investigate this problem by extending the previous experiments to germfree mice

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MATERIAL AND METHODS

Mice Young albino mice both male and female of CD 1 and NMRI strains were used CD 1 mice were obtained germfree (GF) from the Charles River Mouse Farms Wilmington Mass, USA The mice were kept under germfree conditions as described elsewhere (15) Negative cultures during and at the termination of experiments proved that the animals remained GF The mice were 6-8 weeks of age at the time of the initiation of experiments

NMRI specific pathogen free mice were originally obtained from the National Institute of Public Health, Oslo

TABLE 1 *Mortality of Conventional and Germfree Mice Following Intranasal Inoculation of 0 1 Ml Influenza A Virus*

Virus titre	Germfree Dead/Total	Germfree Mean survival days	Conventional Dead/Total	Conventional Mean survival days
10^{4}	4/4*	7 25	4/4	5 75
10^{5}	3/4	11 50	3/4	9 75
10^{6}	3/4	12 75	4/4	10 00
10^{7}	1/4		0/4	
10^{-8}	0/4		0/4	
LD_{50}	$10^{6\ 3}$		$10^{-6\ 3}$	

* Final mortality after 14 days observation

Virus Influenza A_2/Singapore 1/57 strain was given to us by Dr *K Herzberg*, Frankfurt a M, W Germany In our laboratory the virus was passed once in the allantoic cavity of 10 days old embryonated eggs The allantoic fluids were harvested 3 days after inoculation Allantoic fluids with high haemagglutination titres were pooled and stored at –70° C The infectious titre was tested *in vitro* in Vero cells and *in vivo* in mice, by intranasal inoculation of 0 1 ml of the virus

Standard bacterial vaccine The vaccine was purchased from the National Institute of Public Health Oslo The vaccine is composed of formalin killed bacteria suspended in NaCl solution, and contains species regularly isolated from the respiratory tract, total 1 8 × 10^{9} bacteria per ml (4)

Interferon assay Blood was obtained from the axillary vein at times indicated in the text Serum was separated after clotting All samples were adjusted to pH 2 by 1N HCl, and kept at 4° C for two days Before testing the pH was readjusted to neutral by 1N $NaHCO_3$ Interferon activity in the sera was assayed by means of the infectivity inhibition test, employing the micro assay as described in detail elsewhere (2) In brief twofold dilutions of the sera were added to freshly seeded $L\text{-}F_1$ cells in plastic microtrays After incubation overnight the test cups and control cups without serum, were challenged with approximately 10 mean tissue culture infectious doses ($TCID_{50}$) of Vesicular stomatitis virus (VSV) A back titration of the challenge virus was run in parallel in each test After sealing the trays they were incubated at 37° C Microscopical reading was done after 3 days, when the end point titration of VSV was complete The IF titre was estimated as the highest dilution which inhibited viral cytopathogenic effect in 50 per cent of the cups calculated by the method of Reed and Muench, The titres were calibrated to 10 $TCID_{50}$ of challenge virus by means of the standard slope of regression line for mouse IF tested in the $L\ F_1$/VSV system, as described in detail elsewhere (2)

RESULTS

Infectivity of Influenza Virus in GF and Conventional Mice

Mice were inoculated intranasally (i n) with 0 1 ml of tenfold dilutions of influenza A_2 virus without anaesthesia Each dilution was given to 4 mice The inoculated mice were observed for 14 days and mortality was recorded twice daily The mean lethal dose of virus was practically the same in GF and in conventional mice (Table 1) The survival times were generally somewhat shorter in conventional mice than in their GF counterparts given the same virus dose, but the differences were not significant (Wilcoxons signed rank test $0\ 1 > p > 0\ 05$)

Effect of SBV on Influenza Virus Caused Mortality

Twenty GF mice were injected i p with 0 1 ml undiluted SBV Five hours later they were inoculated with 10 LD_{50} influenza A_2 virus Another group of 20 GF mice, and 30 conventional mice received the same virus inoculation but no SBV All mice were observed for 14 days and mortality was recorded twice daily (Fig 1) Total mortality after 14 days in influenza inoculated GF mice was somewhat reduced by SBV treatment from 41 5 to 25 per cent (χ^2 2 780, $0\ 1 > p > 0\ 05$)

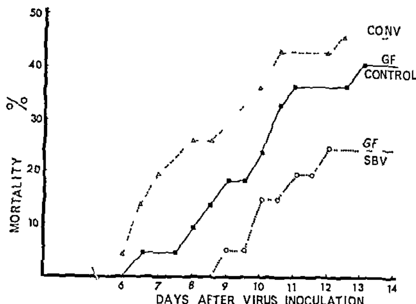


Fig 1 Cumulative mortality of conventional and germfree mice following i n inoculation of 10 LD_{50} influenza A virus and the influence of i.p injected standard bacterial vaccine

The final mortality of influenza inoculated conventional mice was almost the same as that of their GF counterparts, but they seem to die after a shorter lag period than GF mice

Interferon Induction by SBV in GF and Conventional Mice

A group of GF mice was injected i p 0 1 ml undiluted SBV At 2, 4, 6, 8, 24 and 48 hours later 2 mice were removed from the isolator, bleed from the axillary vein, and the IF content of their pooled serum was determined A parallel experiment was done in conventional mice Interferon activity was detected both in GF and in conventional mice (Fig 2) Peak titres were comparable in the two groups, perhaps it was slightly higher in GF mice Peak titres were demonstrated somewhat later in GF than in conventional mice

DISCUSSION

The present data give us some information which supplements the data presented in previous communications The SBV does have a preventive effect on influenza virus infection also in GF mice The extent of this effect is comparable to that seen in conventional animals (1) This finding indicates that the preventive effect of the vaccine is mainly directed against the viral infection and it is not dependent on the factor represented by the possible secondary bacterial invasion An interaction between the infecting viral agent and the resident flora of the animals seems to be of minor importance in this connection

The mechanism of the preventive effect is still not clear IF production was comparable in GF and conventional mice In fact, it is to be expected if IF is important factor in the antiviral effect of SBV, as the prevention is also of the same magnitude Interferon effect on viral infection in GF mice has been demonstrated earlier (12, 13) The IF levels in the present model seems to be too low to represent the entire explanation of the preventive effect of vaccine Exogenous IF with comparable titres does not influence experimental viral infections Doses of viral and nonviral IF induces which gave significant protection against encephalomyocarditis or

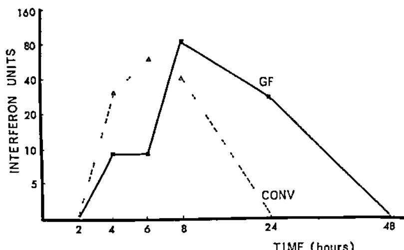


Fig 2 Serum interferon titres in conventional and germfree mice following i p injection of 0 1 ml standard bacterial vaccine

influenza A viruses (9, 16) stimulate levels of circulating IF many times higher than those found in the present experiments Therefore we beleive that additional mechanisms, such as adjuvant effect and non specific stimulation of the immune apparatus may also be in operation

IF production in GF animals has been demonstrated by several authors There is a general agreement that GF animals produce comparable, or slightly higher titres of IF than their conventional counterparts *De Somer & Billiau* (7) found higher titres both in serum, spleen and liver of GF rats following inoculation with *Escherichia coli* endotoxin, compared to conventional animals Using Newcastle disease virus (NDV) as inducer *Fitzgerald & Pollard* (8) demonstrated comparable levels of serum IF but higher titres of spleen and liver IF in GF mice *Havel et al* (11) found higher serum IF levels in GF mice as response to NDV but not to endotoxin, compared to conventional mice An age related IF production during mouse hepatitis virus infection of GF mice was demonstrated by *Lavelle & Starr* (12) IF production increased with age both in liver and in spleen of GF but not of conventional mice Three weeks old GF mice produced less IF one day after virus challenge than conventional mice, while in 5 weeks old animals it was reversed Our findings seem to aggree with the earlier observations IF titres were comparable or possibly slightly higher in GF mice The peak titre was observed somewhat later in GF mice Similar delay was also demonstrated after NDV stimulation (11)

Although the final outcome of infection, mortality, was practically identical in GF and conventional mice, the time of death seems to be different Conventional mice died somewhat sooner after virus challenge Following Sendai virus infection, a similar but more pronounced difference was observed (5) GF mice had lower mortality and longer survival time compared to conventional mice It is reasonable to assume, that the differences are due to secondary bacterial invasion which makes the viral disease worse

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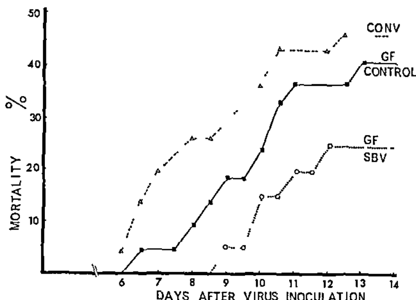


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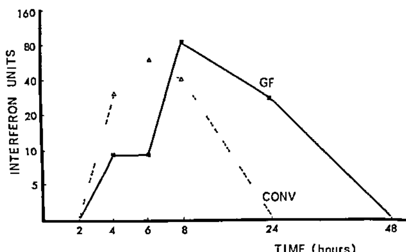


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NITROBLUE-TETRAZOLIUM STAINING OF HUMAN NEUTROPHIL GRANULOCYTES

Effects of Stimulation with Pseudomonas *Antigens in the Presence or Absence of Human Pseudomonas Precipitins*

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Bacteria free filtrates from *Pseudomonas aeruginosa* cultures were added to heparinized blood from 10 patients with multiple Pseudomonas precipitins and from 11 patients without such precipitins before carrying out a nitroblue tetrazolium (NBT) test Increased NBT staining could be induced in neutrophils from all of the patients with demonstrable Pseudomonas precipitins but only in 3 of the patients without precipitins This difference was found to be statistically significant Using patients' plasma but leucocytes from a normal control person it was found that this difference was dependent upon the origin of plasma but independent of the origin of leucocytes These findings indicate that increased NBT staining of neutrophils induced by bacterial products in vitro is in part dependent upon antigen antibody interaction

In 1968 *Park et al* reported increased staining *in vitro* of peripheral neutrophil granulocytes with the histochemical dye nitroblue tetrazolium (NBT) in patients with bacterial infections, and these findings have been confirmed by others (4, 6, 10) The NBT test as outlined by *Park et al* (1968) has subsequently become widely used as a diagnostic aid in febrile disorders It is well established that increased NBT staining follows stimulation of phagocytosis and is dependent upon normal postphagocytic oxydative metabolic activity (2) but the mechanisms in the infected patient responsible for the increased spontaneous NBT staining observed *in vitro* are unexplained Furthermore, it is of some concern that unexpected "false" negative reactions have been reported (3) which would throw serious doubt on the feasibility of this test in clinical routine It therefore seems important to elucidate the mechanisms by which increased NBT staining could be induced during bacterial infections

Increased NBT staining may be induced *in vitro* in neutrophils by exposure of heparinized blood to bacteria free culture filtrates from different genera of bacteria (10) One of us has previously suggested that these culture filtrates contained antigens that could react with antibodies to form immune complexes that subsequently elicited the changes in the neutrophils responsible for increased NBT staining (9) To further test this hypothesis, the following studies were undertaken

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Two groups of patients with cystic fibrosis (CF) were selected on the basis of presence or absence of detectable precipitating antibodies against *Pseudomonas aeruginosa* Bacteria-free culture filtrates from 2 different strains of *Pseudomonas aeruginosa* were then added to heparinized blood from the 2 groups of patients before carrying out the NBT test In order to determine the importance of the origin of the leucocyte, the same experiments were repeated using patients' plasma but leucocytes from a normal control person The results indicate that the increased NBT staining of neutrophils induced by bacterial products *in vitro* is in part dependent upon antigen antibody interaction and independent upon the origin of the leucocytes

MATERIALS AND METHODS

Crossed immunoelectrophoresis (c i e) studies were carried out as described by *Axelsen* (1971) using 1 per cent agarose gel (Batch AGS O58 A, Litex, Glostrup, Denmark) in barbital buffer, pH 8 6, ionic strenght 0 05 The first dimension electrophoresis of 10 µl *Pseudomonas aeruginosa* standard antigen (St Ag (7)) was run for 1 hour at 10 V per cm The second dimension electro phoresis was run for 20 hours applying 1–2 V per cm with patient serum included in the second dimension gel (12 5 µl per cm²) Thickness of gel 1 5 mm dimension of plates 10×10 cm After the run the plates were washed, dried and stained with Coomassie brilliant blue R (Microme no 1137, E Gurr, Ltd London England) as described previously (7)

The *immunodiffusion (i d)* studies were carried out as double diffusions according to the plate technique of *Ochterlony* (1967) The diffusion was carried out in 1 per cent agarose gel (same batch as that used for c i e) in 0 154 M NaCl supported by glass plates 10×10 cm thickness of gel 2 mm The diffusion took place in humid atmosphere at room temperature for 6 days A combined system was used with a central rectan gular trough 60×2 mm containing 0 2 ml of the Pseudomonas filtrate and 20 µl patients serum placed in 4 mm wells at a distance of 5 mm from each other and the central trough After the diffusion the plates were washed dried and stained as outlined for the c i e

The *St Ag* consisted of water soluble con stituents obtained by sonication of 4 different strains of *Pseudomonas aeruginosa* O groups 3 5 6, and 11 The preparation procedure and prop erties of the St Ag have been described previously (7) It contains at least 55 different anti gens (7) and is therefore considered especially well suited for the purpose of revealing Pseudomonas precipitins in human serum. In pilo stud es St Ag was able to induce increased NBT staining in a normal control person in whom demonstrable precipitins against St Ag were absent, indicating that other mechanisms in addition to immune complexes might possibly be able to induce in creased NBT staining (toxins?) This is subject to further studies, but in the present study St Ag was consequently considered less suitable for an elucida tion of the role of immune complexes in the induction of increased NBT response

The *Pseudomonas aeruginosa filtrates* were obtained from 2 strains one strain (designated *P 1*) was isolated from a patient with chronic bronchitis and pneumonia due to this organism This strain has not been O group typed The other strain (designated *P 2*) is identical to one of the four strains used for making the St Ag and belongs to O-group 6 It was isolated from a patient with CF Both strains were obtained from tracheal aspirates and cultured in meat infusion broth enriched with 10 per cent horse serum (*serum broth* Statens Seruminstitut Copenhagen, Denmark) After 24 hours incubation at 35° C the cultures were centrifuged at 1800 × *g* and the supernatants passed through 0 45 µm Millipore® filters and stored in small aliquots at –20° C

The P-1 and P 2 filtrates were compared to St.Ag by means of a rabbit antiserum (St Ab) raised against St Ag (7) St Ab was absorbed with P 1 and P 2 by mixing equal volumes of St.Ab and P 1 or P 2 or 0 154 M NaCl as control The absorption took place at 37° C for 1 hour The results of the absorptions were then evaluated by c i e of 10 µl St Ag against the absorbed St.Ab (10 µl per cm²) according to the above outlined methods for c i e By comparison with the result of the control c i e showing the St Ag–St Ab re ference pattern it was found that both P 1 and P 2 had absorbed antibodies corresponding to 1 of the 55 precipitates visible on the control c.i e and P 1 in addition had absorbed antibodies correspond ing to another of the precipitates This finding indicated that P 1 and P 2 contained antigenic materials corresponding to 2 respectively 1 of the antigens of St.Ag This was confirmed by i d studies No precipitates could be demonstrated by c i e however, when P 1 or P 2 was run against St Ab or patients sera. The reason for this has not been found

Patients Twenty one patients with CF were in cluded The diagnostic criteria have been described previously (7) All patients were examined and followed as out patients every month including bacteriological examination of tracheal secretion (7) In 10 of the patients, designated *CF + Ab* circulating precipitating antibodies against the

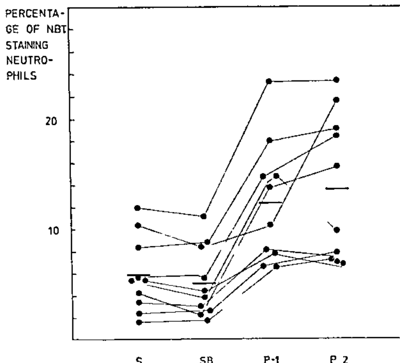


Fig 1 Results of directly stimulated (direct) NBT tests in 10 CF patients containing precipitins against *Pseudomonas aeruginosa* St.Ag in c i e Each patient was tested in four different conditions 1) Spontaneous NBT test (S), – 2) with the culture medium used for the filtrates (serum broth), 5 μl per 0 1 ml blood (SB), – 3) bacteria free filtrate of *Pseudomonas aeruginosa* culture, strain 1, 5 μl per 0 1 ml blood (P-1), and – 4) filtrate from strain 2 (P-2) The results from each patient are connected with lines, and each value represents one NBT test The bars represent mean values of the tests in each condition.

St Ag had previously been detected by c i e All patients in this group had harboured mucoid strains of *Pseudomonas aeruginosa* for at least 2 years (mean 2 5 years) The second group, designated *CF — Ab*, comprised 11 patients in none of whom precipitating antibodies against St Ag had previously been detected *Pseudomonas aeruginosa* had never been isolated from any of the CF — Ab patients Average control period in the clinic was 4 years for CF + Ab and 3 9 years for CF — Ab The mean age was 10 5 years for CF + Ab (range 6 5 to 21) and 9 2 for CF — Ab (range 3 5 to 16 5) CF + Ab included 7 male and 3 female patients and CF — Ab 6 male and 5 female patients Heparinized blood, plasma and serum used in the present studies were obtained simultaneously from all 21 patients who all were afebrile and out patients at the time of examination

The *NBT tests* were carried out according to *Park et al* (1968 with the modifications described earlier (9) For direct stimulation (*direct tests*) 5 μl filtrate was added to 0 1 ml heparinized (approximately 50 i u heparin per ml blood) freshly drawn venous blood from the patients immediately before the NBT tests For stimulation of heterologous neutrophils (*indirect tests*), 0 3 ml freshly isolated heparinized plasma from the patients was pre incubated with 20 μl filtrate for 60 minutes at 35° C Blood cells from 0 3 ml heparinized freshly drawn venous blood from a normal person were washed 3 times at low speed centrifugation (250 × *g*) in Hank's balanced salt solution containing 1 mg gelatin and 19 5 i u heparin per ml pH 7 45 The final cellular pellet was resuspended in the 0 3 ml patient plasma pre-incubated as outlined, and the NBT tests carried out immediately The same normal person was used as donor of heterologous blood cells in all indirect tests His serum contained no detectable precipitating antibodies against the St Ag in c i e or i d, and he had never experienced infection with *Pseudomonas aeruginosa* Direct tests were done with 1) P 1, and 2) P 2 filtrate, 3) without any

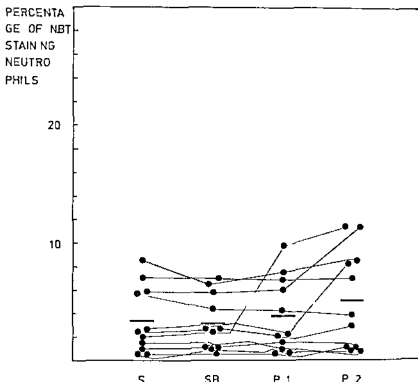


Fig 2 Result of direct NBT tests in 11 CF patients without demonstrable precipitins against the St Ag in c i e Test conditions and symbols as in Figure 1

addition (spontaneous NBT test corresponding to the ordinary NBT tests) and 4) with 5 μl serum broth the last 2 test conditions serving as control values Indirect tests were done with P 1 and P 2 and with serum broth serving as control value

In each test 500 consequtive neutrophils from 2 smears were counted and evaluated in bl nd by one person Statistical calculations were carried out using the Mann Whitney rank sum test and Spearman s correlation coefficient R (Documenta Geigy)

RESULTS

All of the CF + Ab contained multiple precipitating antibodies against the *Pseudomonas aeruginosa* St Ag in c i e The mean number of precipitins was 20 with a range of 4 to 50 In none of the CF − Ab sera could any precipitins against the St Ag be detected All sera were further tested by i d against the P 1 and P 2 filtrates Lines of precipitation could be demonstrated in 9 of the 10 CF + Ab sera against P 2 and in 5 against P 1 None of the sera contained more than 1 demonstrable pre p tin by this method Precipitins against either P 1 or P 2 could not be demonstrated by i d in any of the 11 CF − Ab sera

The results of the direct NBT tests in the 2 groups of patients are given in Fig 1 and 2 Addition of both P 1 and P 2 to heparinized blood from CF + Ab patients caused an increase in the percentage of NBT staining neutrophils compared to the control tests in all 10 patients (Fig 1) Statistical calculations of the results in the CF + Ab group showed that these increases were significant ($p < 0\ 01$) The results obtained with P 1 were significantly correlated to those obtained with P 2 ($R = 0\ 7576$, $p < 0\ 01$)

In the CF − Ab group increased staining could only be induced in 3 patients by P 2 and only in 1 of these by P 1 (Fig 2) In the CF − Ab group as a whole there was no significant effect of stimulation with either P 1 or P-2 compared to control tests ($p > 0\ 10$)

The results of the indirect tests are given

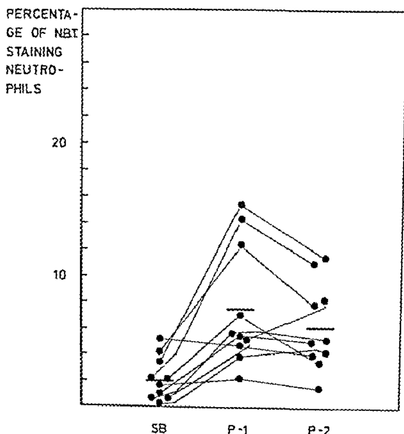


Fig 3 Results of indirect stimulation (indirect tests) of heterologous neutrophils from one normal person with plasma from 10 CF patients containing precipitins against *Pseudomonas aeruginosa* St Ag in c i e Before addition of patients plasma to heterologous neutrophils plasma portions of 0 3 ml were pre incubated with either culture medium used for the filtrates (serum broth), 20 µl (SB), bacteria free filtrate of *Pseudomonas aeruginosa* culture, strain 1 (P 1), 20 µl, or filtrate from strain 2 (P-2), 20 µl The results from each patient are connected with lines and the bars represent mean values of all 10 patients for each test-condition

in Figures 3 and 4 Plasma from 8 of the 10 CF + Ab patients pre-incubated with either P-1 or P-2, caused increased NBT staining in heterologous neutrophils compared to control tests with serum broth (Fig 3) These results too were found to be statistically significant for the CF + Ab group ($p<0 01$) and the results obtained with P-1 were statistically correlated to those obtained with P-2 ($R = 0 7213$, $p < 0 05$) The 2 CF + Ab plasma which did not induce increased staining following pre-incubation with P-1 and P-2 did not differ from that in the rest of the group with respect to precipitins They contained 3 and 7 precipitins in c i e and both had precipitins against P-1, one also against P 2, in i d In the CF – Ab group, plasma from only 3 of the 11 patients pre-incubated with P 2, and one of those also with P-1, induced increased staining in heterologous neutrophils (Fig 4) These were from the patients who also reacted with increased staining in direct tests (Fig 2) In the CF – Ab group as a whole, however, there was no statistically significant effect of pre-incubation of plasma with either P-1 or P-2 compared to control tests ($p > 0 10$) There was no statistically significant relationship between the number of precipitins in the CF + Ab group and the degree of increased

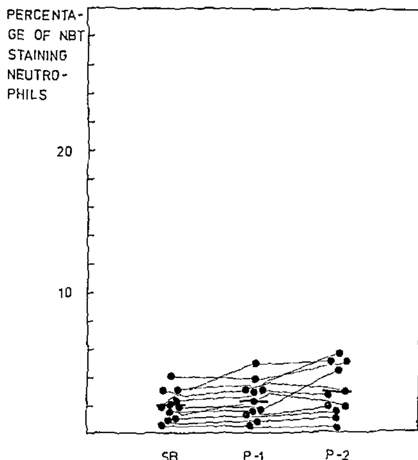


Fig 4 Results of indirect tests using plasma from 11 CF patients without demonstrable precipitins aga nst the St Ag Test conditions and symbols as in Fig 3

NBT staining in either direct or indirect tests It is noteworthy, however, that indirect tests showed the highest degree of increase if plasma from 2 patients containing the highest number of precipitins in c i e was used, i e 39 and 50 lines of precipitation

DISCUSSION

The design of the present study was aimed at a demonstration by means of a sensitive method of circulating precipitins against *Pseudomonas aeruginosa* in patients sera (7) By this method the patients were classed in 2 groups one with such precipitins and one without The correlat n of presence of antibodies to infection with *Pseudomonas aeruginosa* has been discussed previously 7)

These 2 groups were then examined with regard to the effect of 2 bacteria free filtrates of *Pseudomonas aeruginosa* cultures upon the number of NBT staining neutrophils *in vitro* These filtrates were selected from a panel of filtrates derived from different genera of bacteria A number of normal persons and patients suffering from various disorders have been tested in direct tests with this panel and it has been found that some filtrates will induce increased NBT staining in all persons tested, whereas other filtrates will induce increased staining in some but not all persons (9) A similar individual response pattern has also been noted by others (10)

P-1 and P-2 contained 2 and 1, respectively of the 55 antigens present in St Ag but might contain antigens not present in St Ag However, the i d studies showed that 90

per cent of the CF + Ab patients harboured detectable precipitins against P 2 and 50 per cent against P 1, whereas none of the CF – Ab patients harboured detectable precipitins against P 1 or P 2

In the present study it was found that P-1 and P 2 could regularly induce increased staining in neutrophils from the patients with demonstrable circulating antibodies against *Pseudomonas aeruginosa* in c i e and seldom in neutrophils from patients without such antibodies The results of the indirect tests furthermore indicate that this difference was dependent on the origin of the plasma but independent of the origin of the leucocytes since nearly the same results were obtained when patients plasma were pre incubated with the filtrates and thereafter exposed to heterologous neutrophils These findings indicate that the primary event when increased NBT staining is induced *in vitro* by bacterial products is interaction of bacterial antigens with antibodies

Previous studies in this laboratory have indicated that increased NBT staining may be brought about by interaction of bacterial antigens with antibodies with the formation of immune complexes, and that high molecular weight complexes are more effective than low molecular weight in this respect (9) Since the technique employed in the present study for detection of precipitating antibodies is highly sensitive (7) it is interesting that increased staining could be induced in homologous and heterologous neutrophils with blood or plasma from a few patients without Pseudomonas precipitins demonstrable in c i e (St Ag) or in i d (P 1 and P 2) (Fig 2 and 4) The reason for this is not clear one possibility is that low concentrated or non precipitating immune complexes may also induce increased staining Relevant to this is the finding that it has been possible by means of c i e to demonstrate antibodies in concentrated pooled normal human gammaglobulin precipitating with one of the antigens present in the St Ag (8) In unconcentrated normal sera these antibodies are not demonstrable (8) whereas agglutinating antibodies against *Pseudomonas aeruginosa* have been demonstrated in such sera (5)

The present study indicates that interaction of bacterial antigens with corresponding antibodies is able to induce increased NBT staining of human neutrophils *in vitro* and seems to support our previous findings (9) It remains to be studied whether this mechanism is responsible for the increased spontaneous NBT staining seen during bacterial infections If however such a mechanism was operative *in vivo* to induce increased NBT staining the finding of false negative reactions in infected patients might be offered an explanation based upon the absence of antibodies against the invading organism, or conversely absence of bacterial antigens from the blood stream

Several questions should be answered to elucidate the possible interaction of immune complexes with neutrophils as a mechanism inducing increased NBT staining These would include the size concentration and possibly specificity in terms of class of immunoglobulin involved plus the role of heat-labile factors such as complement (9) It is to be hoped that such information might help to explain the wide range of values of NBT tests seen in infected patients and further clarify the usefulness of this test in clinical practice

This work was supported by grants from *The Michaelsen Foundation The Danish Medical Research Council The Foundation for the Advancement of Medical Science The Thorvald Madsen Legat* and *Landsforeningen til bekæmpelse af Cystisk Fibrose* Mrs *Ulla Hasby* and Mrs *Anne Bethsen* are thanked for skilful technical assistance

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ANTIBODY STIMULATION IN INDIVIDUALS WITHOUT DEMONSTRABLE POLIOVIRUS ANTIBODIES FOLLOWING A FIFTH INJECTION OF INACTIVATED POLIOVIRUS VACCINE

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The effect of a fifth dose of inactivated poliovirus vaccine was estimated in a group of recruits belonging to the age groups comprising the individuals in Sweden who received the first, less effective poliovirus vaccines As a rule they had received their primary immunization (3 shots) during the years 1957-59 and a fourth injection around 1963 to 64 The response to the fifth dose given in 1971 indicated that they responded well to the antibody stimulus Even individuals without demonstrable antibodies prior to the booster reacted to a high degree with an antibody rise similar to that of a secondary response

In Sweden, only inactivated poliovirus vaccine has been used The routine immunization comprises 4 injections The first two doses are given about 1 month apart, a third is administered after 8-12 months and a fourth about 4 years later In 1968, ten years after the introduction of vaccinations a surveillance of the general sero immunity to the disease was performed (1) The results of this investigation suggested that the age groups which initiated their immunizations in 1957 to 1959 possessed an immunity lower than that in the rest of the population These early vaccines were of poorer quality than those used later The age groups concerned comprised children and young adults born between 1948 and 1958 (Table 1) In 1971, it was decided to study the response to a fifth booster dose by those vaccinees which lacked demonstrable antibodies

TABLE 1 *Percentages of Persons Lacking Antibodies to One or Several Types of Poliovirus The Number of Individuals Lacking Antibodies is Correlated to the Total Number with the Same Vaccination History The Dates Represent the Year of Initiation of the Immunization*

Vaccination history	Children %	Adults %
Unvaccinated	77	43
2*-3 injections 1957 60	23	
4 ,	15	
2*-3 , 1961 66	5	4
4 ,	1	
1 2 , 1967	26	
Data unknown	14	

* 1 2 persons in these groups had only 2 injections

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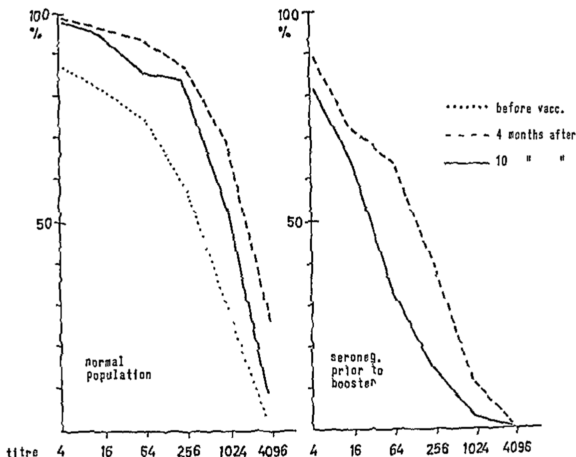


Fig 1 Accumulated titre distribution (immunologic profiles) to poliovirus type 1
Left Randomly selected group of previously vaccinated recruits (80 individuals)
Right Recruits previously vaccinated but lacking demonstrable antibodies to type 1 in their pre booster serum sample (27 individuals)

MATERIAL AND METHOD

Introductory Investigation

One regiment comprising 750 recruits was chosen for the investigation, which was initiated in the summer of 1971 The recruits were born mainly in 1950 to 1951 and almost all were vaccinated according to the routine procedure beginning in 1957 to 1959 The men were parenterally vaccinated and blood samples were obtained by venipuncture Antibody titrations on sera were performed by use of immuno-inactivation of a standard virus preparation and inoculation into tissue-culture tubes The cytopathogenic effects were scored as described earlier (2) The lowest serum d[illegible]tion tested was 1 4 The results of the titrat[illegible]n [illegible]howed that 14 per cent, 2 per cent and 23 pe[illegible] [illegible] of the recruits lacked demonstrable antibodies [illegible]ovirus type 1, 2 and 3, respectively

Continued Examinations of the Recruits

For the continued evaluation of the antibody response to the fifth injection the following 3 groups were constructed

- Group 1 A randomly selected group (100 individuals)
- Group 2 Type 1 negative recruits (30 individuals)
- Group 3 Type 3 negative recruits (100 individuals)

Attempts were made to collect blood samples 4 and 10 months after the vaccination Such samples were obtained from 80 recruits in group 1, from 27 in group 2, and from 71 in group 3

RESULTS

The antibody levels 4 and 10 months after the booster are illustrated by "immunological

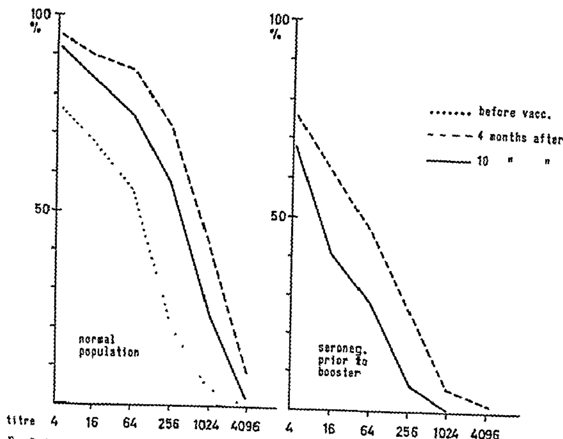


Fig 2 Accumulated titre distributions (immunologic profiles to poliovirus type 3
Left Randomly chosen group of previously vaccinated recruits (80 individuals)
Right Recruits previously vaccinated but lacking demonstrable antibodies to type 3 in their pre-booster serum sample (77 individuals)

profiles" (accumulated titre distributions) in Figs 1 and 2 In Fig 1 the "immunological profiles" to poliovirus type 1 of groups 1 (left) and 2 (right) are shown It can be noted that 99 per cent of the randomly chosen population of recruits had demonstrable antibodies to type 1 ten months after the booster while the corresponding figure was 83 per cent among the vaccinees seronegative to type 1

In the randomly chosen recruits in group 1, type 3 antibodies persisted in 94 per cent after 10 months (Fig 2), while 68 per cent of the pre-vaccination type 3 negative recruits (group 3) were seropositive A few samples were collected also from recruits who had never received poliovirus vaccine They were seronegative both on the occasion when they received this single dose and 4 months after.

Effect of Treatment with Mercapto-ethanol

Fifty of the serum samples collected after the booster, the majority containing titres not higher than 1 to 64, were titrated before and after treatment with mercapto-ethanol. The method described by *Svehag & Mandel* (3) was used The treatment as a rule selectively destroys the IgM component A purified IgM preparation containing poliovirus antibodies kindly supplied by Kabi AB, Stockholm, Sweden, was used as a control Titres of untreated and treated samples are correlated and illustrated in Fig 3 No significant reduction of titres was observed between un-

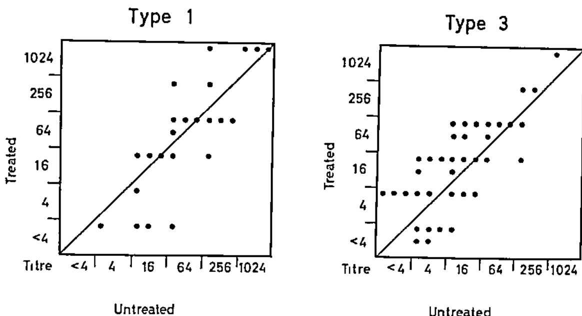


Fig 3 Correlation between antibody titres to poliovirus type 1 (left) and 3 (right) prior to and after treatment with mercapto-ethanol

treated and treated Only a few samples with lower titres may be suspected to possess only IgM antibodies

DISCUSSION

The results of this study indicate that the majority of the 4 times immunized individuals were likely to possess immunologic memory Most of those individuals who did not exhibit any demonstrable antibodies to poliovirus reacted to the fifth injection in a fashion similar to the reaction to a "booster reaction" Earlier studies had shown that a single injection in totally non immune individuals seldom resulted in antibodies persisting after 10 months (4) Thus, a single extra injection to the seronegative recruits who had received 4 injections earlier appeared quite effective in inducing presisting antibodies and any further immediate reinforcement does not seem to be necessary The remaining antibodies appeared mainly to be of the mercapto-ethanol resistant IgG type

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INFLUENCE OF AZATHIOPRINE ON HUMORAL DEFENCE FACTORS AGAINST *ESCHERICHIA COLI* IN GERMFREE AND MONOCONTAMINATED RATS

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Kaptein Wilhelm Wilhelmsen og Frues Bakteriologiske Institutt, University Hospital, Rikshospitalet, Oslo, Norway

By means of radioisotope technique the influence of azathioprine on humoral defence factors against *E coli* was studied on germfree and ex germfree rats monocontaminated with *E coli* Using inactivated sera, by the addition of serum from germfree rats as a source of complement, titration of antibodies showed that the bactericidal activity of serum from azathioprine treated rats was reduced in relation to control serum By distinguishing the immunoglobulins on the basis of their sensitivity to 2 mercaptoethanol it was found that azathioprine blocked the production of 2 mercaptoethanol resistant antibodies The complement activity in the azathioprine treated animals was not found to be decreased Apart from complement other non specific humoral factors against *E coli* did not seem to participate significantly in the system used

Azathioprine is regularly used in combination with corticosteroids in order to enhance survival of allografts These drugs have, however, serious adverse effects among which increased susceptibility to infections remains a main problem of clinical allotransplantation today

The infection enhancing effect of the drugs used, might be due to impairment of cellular or humoral host defence factors The most important antimicrobial functions of these humoral factors are the opsonic and bactericidal activities mainly mediated by specific antibodies and complement components (8, 13, 16)

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A suppressed antibody formation following antigenic challenge has been observed in man and animals simultaneously treated with purine antagonists (5, 6, 9, 10, 11, 12) How this treatment affects complement activity, however, has not been thoroughly investigated (2)

Germfree animals monocontaminated with a test microbe and kept in a controlled environment, appear to be suitable for studying the effect of immunosuppressive drugs on specific antibody formation (8) In addition, germfree animals appear suitable for the evaluation of drug effects on the production of nonspecific humoral factors involved in the defence against the microbe used

Using germfree rats and ex germfree rats monocontaminated with *E coli*, the aim of the present study has been to evaluate the

influence of treatment with azathioprine on antibody formation, and on the production of nonspecific humoral factors participating in the opsonic and bactericidal activities towards *E coli* These activities of sera were tested in vitro according to a previously described radioisotope technique (14)

MATERIALS AND METHODS

Animals

Germfree (GF) rats of the CDF strain (Charles River Breeding Labs, Wilmington Mass USA) were reared as described elsewhere (7) Twenty one animals at 2 3 months of age were used without regard to sex and divided at random into the following groups i) 5 GF rats ii) 4 GF rats treated with azathioprine (GFA), iii) 6 rats monocontaminated with *E coli* (M), and iiii) 6 monocontaminated rats simultaneously treated with azathioprine (MA)

Monocontamination

Contamination of the GF rats was performed as previously described (7) with the same strain (X7) of *E coli* as used in the in vitro test (14)

Drug Treatment

Azathioprine (Imuran®) in aquous solution was injected intraperitoneally (i p) in daily doses of 40 mg per kg for 14 days in the GFA and MA groups of rats The drug was kindly supplied by Burroughs Wellcome Ltd as a dry substance in sterile glass ampules The ampules were introduced into the isolators according to the germfree technique as previously described (7)

Serum

Twenty four hours after the last medication the rats were removed from the isolators and blood collected by heart puncture Sera from rats within the individual groups were pooled and stored at 20° C

Source of Complement

In experiments with heat inactivated sera tested for antibody activity a constant amount (5 per cent) of serum from GF rats was added as a source of complement

Inactivation of Serum

was obtained by heating a 56 C for 30 minutes

Determination of Immunog l d r c

Mercaptoethanol treatmer a s bsequent alkylation by iodacetamide was car accord i g

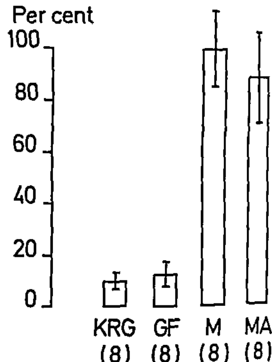


Fig 1 Phagocytosis by PMN Uptake of ³²P labelled *E coli* incubated in medium without serum (KRG) and by addition of different sera i e GF from germfree rats M from ex germfree rats monocontaminated with *E coli* and MA from monocontaminated rats treated with azathioprine The mean uptake rate per mg cell protein in the presence of M serum is set as 100 per cent Brackets indicate number of observations
I – ± 1 SD

to *James & Medawar* as described by *Jakobsen* (6) with modifications A Krebs Ringer phosphate buffer with 10 mM glucose (KRG) was used instead of Hank s solution during treatment of serum with equal volume of 0 2 M 2 mercaptoethanol (2 ME), and also during the dialysing procedure *instead of the phosphate buffer and NaCl solution* prescribed The dialys s was carried out at + 4° C.

Determination of Phagocytosis and Release of Label from Bacteria into Medium

were carried out as previously described (14) The opsonic activity of serum was studied by measuring its ability to induce uptake of ³²P labelled *E coli* into polymorphonuclear neutrophils (PMN) which were suspended in media containing 5 per cent of the serum to be tested The uptake of radioactivity into PMN was related to cell protein (14)

Bactericidal activity of serum was measured indirectly using release of label as a parameter of

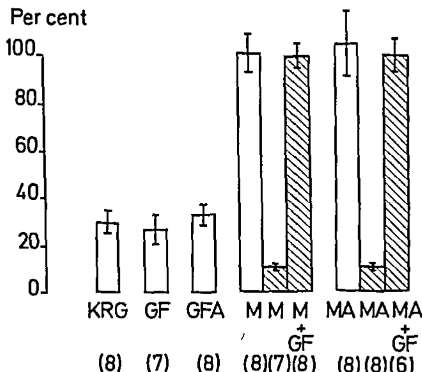


Fig 2 Release of label from ^{32}P labelled *E coli* suspended in medium without serum (KRG) and by addition of different sera i e GF from germfree rats GFA from germfree rats treated with aza thioprine, M from ex germfree rats monocontaminated with *E coli* MA from monocontaminated rats treated with azathioprine Rates of release are also figured out for M and MA serum after inactivation by heat and for inactivated sera after addition of GF serum The rate of release in the presence of M serum is set as 100 per cent Brackets indicate number of observations I = ± 1 SD

the bactericidal effect of the 5 per cent serum present

In the quantitative tests on the antibody activity of serum from monocontaminated rats serum dilu tions down to 1 100 000 were used

Statistical Analysis

Each experiment was carried out at least twice The two samples ranks test was used for statistical analysis (15)

RESULTS

Phagocytosis

The rates of uptakes of ^{32}P labelled *E coli* into PMN are presented in Fig 1 The uptake rate in the presence of serum from rats monocontaminated (M) with *E coli* was set as 100 per cent, which was 10 times the uptake rate when medium without serum (KRG) was used The addition of GF serum to the KRG medium did not result in a higher uptake rate than in the absence of this serum ($p > 0\ 10$) Sera from mono-contaminated rats simultaneously treated with azathioprine (MA) resulted in an uptake rate which was 89 per cent of that induced by serum from untreated, monocontaminated rats The difference was, however, not statisti cally significant ($p > 0\ 10$)

Release of Label into Medium

In Fig 2 the release of label from bacteria into the medium is shown The

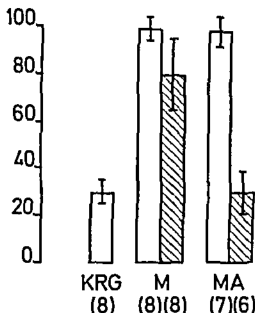


Fig 3 Release of label from ^{32}P labelled *E coli* suspended in medium without serum (KRG) and in the presence of crude sera and sera treated with 2 mercaptoethanol M — serum from ex germfree rats monocontaminated with *E coli*, and MA = serum from monocontaminated rats treated with azathioprine The rate of release in presence of crude M serum is set as 100 per cent Brackets indicate number of observations
I = ± 1 SD

release in the presence of serum from monocontaminated rats (M) was set as 100 per cent, which was more than 3 times the rate of release when medium lacking serum (KRG) was used When serum from GF rats or GF rats treated with azathioprine (GFA) was added to the medium, the rate of release was not significantly different from that obtained in the absence of serum ($p > 0.10$ and $p > 0.10$ respectively) Sera from azathioprine treated rats in the monocontaminated group (MA) resulted in a release of label which was not different from that when serum from untreated monocontaminated rats was used ($p > 0.10$)

Inactivation of sera reduced the bactericidal activity of M and MA sera below the KRG level The bactericidal activities of these sera were completely restored by the addition of 5 per cent GF serum as source of complement ($M \approx$ inact $M + GF$, $p > 0.10$) as shown in Fig 2

Mercaptoethanol Treatment

The sensitivity of different sera to treatment with 0 2 M 2-ME is presented in Fig 3 in relation to levels of the respective sera when not treated with 2 ME (crude serum) All sera were inactivated and GF serum then added

Following the 2-ME treatment the M and MA serum behaved entirely differently ($p < 0.01$) The M serum still induced 80 per cent release of label from bacteria, while the activity of MA serum was de

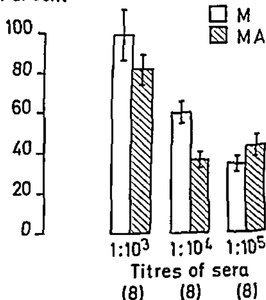


Fig 4 Release of label from ^{32}P labelled *E coli* in the presence of a standard amount (5 per cent) of GF serum and by the addition of different titres of inactivated sera, i e M from ex germfree rats monocontaminated with *E coli* and MA from monocontaminated rats treated with azathioprine The rate of release induced by titre 1 1000 of M serum is set as 100 per cent Brackets indicate number of observations
I = ± 1 SD

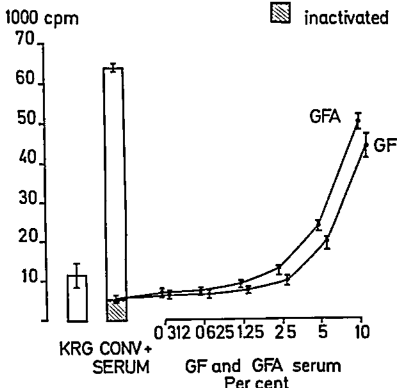


Fig 5 Release of label from [32] P labelled *E coli* suspended in medium without serum (KRG) and in the presence of serum from conventional rats Rates of release induced by the latter serum are figured out before and after inactivation by heat, and after addition of increasing amount of serum from germfree rats (GF) and serum from germfree rats treated with azathioprine (GFA) to the standard amount of inactivated serum from conventional rats I – ± 1 SD

creased to the level of medium without serum (KRG)

Quantification of Antibody Activity

The bactericidal activity of tenfold dilutions of sera from the monocontaminated rats is shown in Fig 4 At the titre 1 1000 the MA serum was less active than the M serum ($p < 0.05$), a difference which was even more significant at titre 1 10 000 ($p < 0.01$) By further dilution the bactericidal activity of both sera was reduced to the GF level

Complement

The ability of GF serum to restore the bactericidal activity of inactivated serum is shown in Fig 2 A comparison of the total complement activity of GF serum and GFA serum (from GF rats treated with azathioprine) is illustrated in Fig 5 To inactivated serum from conventional rats was added complement from these two sources in increasing amounts The bactericidal activity of the inactivated serum was raised to the same extent by either source of complement, i e the activity of complement in GFA serum was not found to be decreased in relation to that of GF serum

Other Nonspecific Humoral Factors

As shown in Fig 1 and Fig 2 the rates of phagocytosis and bactericidal activity were not significantly higher in the presence of GF serum than the rates of the corresponding activities obtained in the absence of serum ($p > 0.10$)

DISCUSSION

The aim of the present study has been to evaluate the influence of azathioprine treatment on rat serum factors easily measured in the in vitro model used, the sensitivity of which is considered high (1, 14)

By varying the specific antigenic stimulus from almost zero in GF animals to a substantial degree in rats monocontaminated with the test microbe, a high degree of specificity has also been achieved in the in vivo model (8)

As a source of complement, sera from GF rats have been used These sera contain negligible amounts of specific antibodies towards *E coli* In addition, errors due to the use of serum from other species are avoided

When GF sera were tested, neither opsonic nor bactericidal activity was found to exceed the level of KRG medium significantly From these findings it seems unlikely that non specific humoral factors apart from complement participate in the activities tested in the system used

The present results indicate that GF serum posesses sufficient complement activity to restore the bactericidal capacity of inactivated serum containing sufficient amounts of specific antibodies On the basis of this observation the effect of azathioprine administration on complement activity in GF rats was evaluated No impairment of complement activity was found in these rats

Midtvedt & *Trippestad* (8) found in serum from GF rats monocontaminated with *E coli* X7 that the opsonic and bactericidal activity was respectively 3 and 4 times higher than in GF serum as early as 10 days of monocontamination These findings indicate that monocontamination with *E coli* induces a rapid synthesis of specific humoral factors, and that the potential of synthesis of such factors is large in GF rats Furthermore, when 10 days of monocontamination were sufficient to demonst[illegible]te [illegible] substantial rise of opsonic and bacterici[illegible] ac[illegible]ties 24 days of azathioprine treatment [illegible] e sufficient to evaluate any drug inhib[illegible] [illegible] he formation of factors responsible for [illegible] tivities

In rats *Currey* (4) found that azathioprine suppressed the primary antibody response to sheep erythrocytes with a dosage exceeding 50 mg per kg given daily i p for 7 days However, the amount used corresponded to the LD 50 (50–75 mg per kg), which is not an ideal dosage for the evaluation of drug effects on antibody formation Present preliminary studies with azathioprine administration to rats have shown that 60 mg per kg per day approximated the LD 50 when given i p for 21 days Therefore, the dose 40 mg per kg was selected and tolerated, although the weight figures might indicate a toxic effect (3)

The present results showed that sera from all monocontaminated rats, treated and untreated with azathioprine, induced a rate of phagocytosis which was 10 times higher than the one induced by GF sera while the bactericidal activity of sera from monocontaminated rats was nearly 4 times higher than that of GF serum Sera from azathioprine treated rats seemed to contain sufficient amounts of bactericidal serum factors to induce a full scale reaction Following 2 ME treatment, however, sera from azathioprine treated monocontaminated rats did not induce complement dependent bactericidal activity, while sera from untreated monocontaminated rats retained 80 per cent of their bactericidal activity in the presence of complement Therefore, the antibodies responsible for bactericidal activity in azathioprine treated rats were found to be exclusively mercaptoethanol sensitive, presumably IgM (6 9), the IgG production being blocked

At the standard concentration of serum (5 per cent) used in the test system, it seems that IgM is able to compensate for the lack of IgG, the opsonic and bactericidal activities against *E coli* being apparently *intact* By dilution of antibodies, however, it could be demonstrated that these activities were actually reduced by azathioprine in relation to sera from untreated control animals It seems reasonable to assume that the qualitative and quantitative changes in specific antibacterial serum activity found in the present study

might be of importance in the defence against invading microorganisms

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BRIEF REPORTS

IgE SERUM LEVELS IN LEPROSY

Jan A J Grabosz, Holger Derblom and *Tore Godal*

Levels of immunoglobulin A and G have been reported to be raised in low resistant (lepromatous) leprosy as compared to levels in high resistant (tuberculoid leprosy, while IgM and IgD have not been found to be significantly raised (2, 3, 6, 7) except in one study (5)

In this study we measured the concentration of IgE in sera from lepromatous and tuberculoid leprosy patients as compared to sera from two different control groups

Materials and Methods

Test subjects Forty nine adult lepromatous (LI + LL) and 52 tuberculoid (TT + BT) patients were included in this study Diagnosis was based on combined clinical and histopathological examination

Two control groups were examined The first consisted of 19 healthy Ethiopian staff members at Princess Zenebework Memorial Hospital, Addis Ababa The second consisted of 19 subjects classified as healthy household contacts of the leprosy patients Serum was collected from defibrinated blood, stored and transported frozen (−20° C) by air to Pharmacia AB, Uppsala, Sweden, for IgE determinations.

Quantification of IgE IgE was determined by a radioimmunosorbent technique, Phadebas IgE Test, Pharmacia AB, Uppsala The principles of the test are presented by *Wide* & *Porath* (8), and also described by *Johansson et al* (4)

Results and Comments

The comparison of IgE values in the four groups of patients shown in Table 1 was done by Kruskal Wallis non parametric one way analysis of variance

The difference between groups was found to be statistically significant ($p < 0.01$) The group consisting of staff members seems to have the highest evidence to differ from the other groups, at least at the low levels The difference between the three other groups in this series is not significant but using Mann-Whitney rank sum test for unpaired data we obtained a 't' value of 1 93 ($0.10 > p > 0.05$) by comparing the group of lepromatous patients with that comprising patients with leprosy It is therefore possible that a significant difference could be found in a numerically larger series

The difference between the two control groups is most likely due to different socio-economic backgrounds and thereby perhaps to differences in exposure to intestinal parasites which are known to give rise to high levels of IgE (1)

TABLE 1 *Results of IgE Determinations*

	No of patients	Median	95 per cent confid limits for median	Range
Staff members	19	668 U/ml	275–2,215	64– 8,250
Household contacts of leprosy patients	19	1,830 U/ml	379–6,050	167–14,300
Lepromatous leprosy	49	2,700 U/ml	2,115–3,785	163–17,750
Tuberculoid leprosy	52	1,690 U/ml	1,005–2,530	100–18,500

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Armauer Hansen Research Institute * *Addis Ababa, Ethiopia & Pharmacia AB Uppsala Sweden*

* Affiliated with All-Africa Leprosy & Rehabilitation Training Centre (ALERT), Addis Ababa and The University of Bergen, Bergen, Norway

Conclusion

In this study, IgE serum levels in lepromatous leprosy patients do not differ significantly from those in patients with tuberculoid leprosy or from healthy leprosy household contacts However, the staff members whose socio economic backgrounds differed had significantly lower IgE levels in serum ($p < 0.01$) The difference is most likely dependent on different exposure to intestinal parasites which are known to give rise to high levels of IgE This study shows also the importance of control groups which, as regards socio-economic background, must be closely matched whenever IgE serum levels are to be studied in developing countries such as Ethiopia

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CROSSED IMMUNOELECTROPHORETIC CHARACTERIZATION OF HERPESVIRUS HOMINIS TYPE 1 AND 2 ANTIGENS

Bent Faber Vestergaard

Herpesvirus hominis (HVH) antigens have been demonstrated by immunodiffusion tests (4, 5, 6) and by immunoelectroosmophoresis (2) This report describes the application of the crossed immunoelectrophoretic method (1) in the analysis of HVH type 1 and 2 antigens The primary aim of this work was limited to enumeration and electrophoretic characterization of the antigens involved

HVH type 1 (MacIntyre) and HVH type 2 (MS) used for immunization of rabbits were produced in a continuous rabbit cornea cell line (3), without serum in the maintenance medium Packed infected cells were disintegrated by ultrasonic treatment (18,000 cycles for 30 sec), mixed with equal amount of incomplete Freund's adjuvans and injected intracutaneously (2 × 0 1 ml) into rabbits once a month for a period of 12 months The standard anti HVH type 1 and 2 antibody preparation used in the present study, was made from a serum pool of 10 rabbits (5 immunized with HVH type 1 and 5 immunized with HVH type 2) by ammonium sulfate precipitation and ion exchange chromatography (5 c) The sera were pooled *in order to obtain an antibody preparation* with broad activity towards all HVH type 1 and 2 antigens

HVH antigens used for immunoelectrophoresis were made by the following standard technique Monolayers of Hep 2 cells (a total of 10^3 square centimeters) were infected with a multiplicity of 5 The cells were scraped off the glass 24 hours later, packed, and 1 ml of a 0 01 M Tris/HCl buffer, pH 7 6, with 10 per cent Triton X 100 was added This gave approximately 2 ml with 10^9 cells per ml The mixture was sonicated at room temperature at 18,000 cycle 3 × 30 sec with 10 minute intervals followed by centrifugation at 100 000 *g for one* hour 20 per cent v/v Glycerol was added to the supernatant, which was stored at −75° C No degradation of the antigens was demonstrated by crossed immunoelectrophoresis after 3 months

The electrophoresis was carried out on 10 × 10 cm glass plates in 1 5 mm thick 1 per cent (w/v) agarose gel (Batch AGS 079, Litex Glostrup) The gel was dissolved in barbital buffer, pH 8 1 ionic strength 0 05 with 0 01 per cent sodium azide First dimension electrophoresis was performed in the presence of 1 per cent (v/v) Triton X 100 (B e & Berntsen, Copenhagen), *applying* 10 volt per cm for 1½ hours Second dimension electrophoresis [illegible]

By running series of crossed immunoelectrophoreses with varying amounts of the standard anti HVH type 1 and 2 antibody preparation in the second dimensional gel, a total of 11 prec p t es with HVH types 1 and 2 was detected Fig 1 plate A shows the immunoelectrophoretic pattern of HVH type 1 (MacIntyre) and plate B HVH type 2 (MS) The relative migration velocity (RMV) was *determined by adding* human albumin to the HVH antigen and antihuman albumin to the standard antibody preparation (5 µg human albumin, electrophoretically pure Behringwerke AG, per well, and 1 µl rabbit anti human albumin Dakopatts A/S, Copenhagen per square cm second dimensional gel) The anod c migration veloc ty of each antigen could then be expressed in percentage of the human albumin migration on the same plate

The reproducibility of the precipitate pattern was determined by running identical plates and measuring the RMV for each prec p tate. The reproducibility of the antigen production technique *was studied by running different* batches of ant gen prepared from the same virus Fig 1, plate C shows a tandem crossed immunoelectrophoresis when HVH type 1 and 2 preparations have been electro [illegible] onal gel (5 d) [illegible] amounts o [illegible] it was possi ble to make a preliminary estimation concern [illegible]

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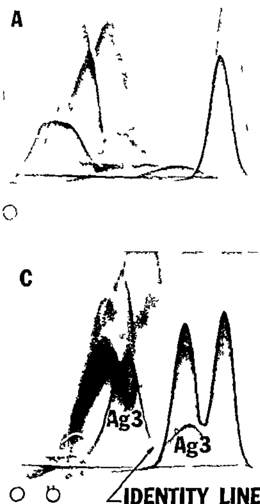


Fig 1 A 15 µl HVH type 1 antigen B 15 µl HVH type 2 antigen, C 15 µl HVH type 1 in right well and 15 µl HVH type 2 in left well A and B with 6 µl and C with 3 µl rabbit anti HVH type 1 and 2 antibody preparation per square cm second dimensional gel Anode was to the right in the first dimensional run and at the top in the second dimensional run Staining Coomassie brilliant blue®

antigenic identity and non identity between the antigens from HVH type 1 and 2 preparations (5 a 5 b)

After identification experiments the individual precipitates were given numbers as indicated in Fig 2
to the
and 8
It is
fact that it had different migration velocity in HVH type 1 and 2 preparations demonstrated the reaction of antigenic identity in tandem electrophoresis (Fig 1 plate C) The presence of two fast moving antigens (Nos 1 and 2) in HVH type 2 preparation only (Fig 1 plate B) was another striking observation

The antigenic characteristics of HVH type 1 and 2 preparations listed in Table 1 were not only representative of the MacIntyre strain and the MS strain but were found also in preparations made from two HVH type 1 isolates and two HVH type 2 isolates typed in this laboratory

The relationship between antigens Nos 6 and 8 needs to be further analyzed The precipitation pattern of the HVH type 1 preparation indicates a partial identity between the two antigens as the anodic leg of peak 8 and the catodic leg of peak 6 are not clearly demonstrated

Crossed immunoelectrophoresis of control antigen (non infected Hep 2 cells prepared by the standard technique) revealed that the precipitate, designated B in Fig 2, and closely resembling the reference human albumin precipitate was of r
viral origin The precipitate was id
bovine albumin by crossed immu
of bovine serum against the st

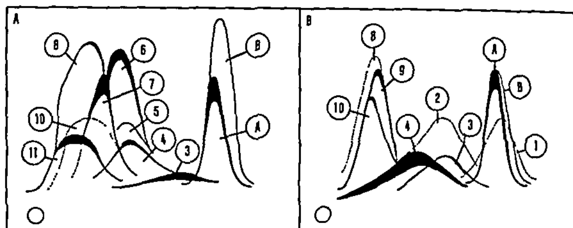


Fig 2 Drawing of Fig 1 A and B with enumeration of the precipitates

TABLE 1. *HVH Type 1 and 2 Antigens Enumerated According to the Anodic Migration Velocity Beginning with Antigen No 1 in the Anodic End, and Ending with Antigen No 11 in the Catodic End of the First Dimensional Electrophoresis*

Number of antigen	HVH type 1 RMV* Median	HVH type 1 RMV* Range	HVH type 2 RMV Median	HVH type 2 RMV Range
1			100	(106–96)
2			70	(73–63)
3	80	(82–76)	70	(72–65)
4	50	(53–45)	50	(54–46)
5	48	(49–47)		
6	46	(48–44)		
7	38	(42–35)		
8	30	(32–27)	30	(33–27)
9			28	(32–24)
10			26	(29–22)
11	21	(24–15)		

* Relative migration velocity (RMV) is expressed as percentage of human albumin migration velocity RMV median and range were determined from 8 individual electrophoreses (4 using the same antigen batch and 4 using different antigen batches of the same virus)

antibody preparation The presence of precipitating antibodies towards bovine albumin was due to the fact that the HVH infected rabbit cells used for immunization, had been grown with 10 per cent bovine serum prior to inoculation with virus A very faint precipitate with an RMV of 75 was also shown to represent a bovine serum component No precipitates could be related to cellular antigens from SIRC cells and Hep 2 cells

I thank Laboratory Technician *Bente Iversen* for excellent technical assistance and *Agnete Ingild*, *Ole Bjerrum* and *Nils Holger Axelsen*, The Protein Laboratory, University of Copenhagen, for inspiration and useful suggestions

References 1 *Axelsen, N H*, *Kroll, J* & *Weeke, B* (Eds) Scand J Immunol 2 Suppl No 1, 1973 a) *Axelsen, N H* ibid pp 91–94 b) *Bock, E* & *Axelsen, N H* ibid pp 95–99 c) *Harboe, N* & *Ingild, A* ibid pp 161–164 d) *Kroll, J* ibid pp 57–59 —2 *Jeansson S* Appl Microbiol *24* 96–100, 1972 —3 *Leerhoy, J* Science *149* 633–634, 1965 —4 *Schneweis, K E* & *Nahmias, A J* Z Immun Forsch *141* 471–487, 1971 — 5 *Thouless, M E* J gen Virol *17* 307–315, 1972 —6 *Watson, D H* J gen Virol 4 163–168, 1969

ERRATUM

The authors, *Kurt Osther* & *Ryan Linnemann*, of the article "Immunofluorescence measurement of C1 inactivator (alpha 2 neuraminoglycoprotein) activity of the surface of human carcinoma cells', which appeared in Acta Pathologica et Microbiologica Scandinavia, Section B, *81* 365, 1973, have informed the Editorial Board that by an unfortunate mistake the work was issued from the Department of Pathology, The Finsen Institute, Copenhagen, instead of from The Warwara Larsen Foundation Complement Laboratory, Copenhagen

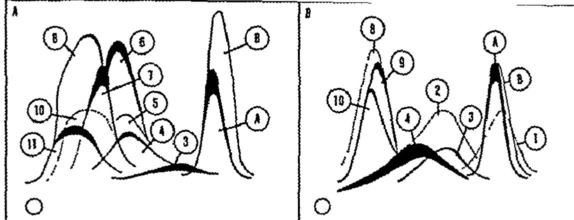


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	Median	Range	Median	Range
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